STUDIES ON HYALURONIC ACID IN SYNOVIAL FLUIDS

By

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Reprinted from
ACTA SOCIETATIS MEDICORUM
UPSALIENSIS
Upsala Läkareförenings Förhandlingar
VOL. LVIII NOS. 3–4, 1953
(Pag. 113–238)

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From the Institute of Medical Chemistry, University of Uppsala, Sweden

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CHAPTER I

Introduction

The highly viscous component of the synovial fluid and its changes in pathological conditions have aroused interest for many years (see the monograph of Kling, 1938). Little progress was, however, achieved in this field until the nature of the main carbohydrate of the 'synovial mucin' was clarified. In 1939, Meyer and his co-workers isolated a polysaccharide from synovial fluid of cattle and man, which proved to be hyaluronic acid. This polysaccharide had previously been prepared from vitreous humour by Meyer and Palmer (1934), and had been characterized as a polymer of high molecular weight composed of N-acetyl-glucosamine and glucuronic acid in equimolar proportion (Meyer and Palmer, 1936).

The long chain nature of the hyaluronic acid molecule was clearly demonstrated by Blix and Snellman (1945) on the basis of viscosity and birefringence studies on hyaluronate from vitreous humour, synovial fluid, and umbilical cord. The polysaccharide was presumed by these authors to occur in its native state in a polydisperse form, and to have a minimum molecular weight of about 200 000—500 000.

The detailed structure of hyaluronic acid has not been definitely established. The results of recent structural studies (Blix, 1951 A; Jeanloz and Forchielli, 1951; Weissmann and Meyer, 1952) appear consistent with the idea that the hyaluronic acid is in the main an unbranched polymer formed from dimer units composed of N-acetyl-glucosamine and d-glucuronic acid. X-ray diffraction pictures have, however, failed to reveal a regular structure (Blix and Snellman, 1945; Kaye and Stacey, 1951). The naturally-occurring hyaluronic acid is easily depolymerised by dilute alkali to a product with a molecular weight of the order of 40 000. Acid groups are formed during this depolymerization. These observations may suggest the presence of other bonds than the glycosidic ones in naturally-occurring hyaluronic acid (Blix and Snellman, 1945; see also Meyer, 1948). Slight differences in chemical composition may exist between hyaluronates of high and low mean polymerization (see discussion, chapter VI).

The interest in hyaluronic acid was greatly increased when Chain

and Duthie (1939) established the enzymatic nature of the 'spreading factor' from mammalian testis (Duran-Reynals, 1928; McClean, 1930). The purified testicular factor was found to hydrolyse 'mucin' from synovial fluid and vitreous humour, causing rapid decrease in viscosity, and later liberation of reducing sugar. In the next few years hyaluronidases were isolated from various other sources, and the identity of the testis factor with hyaluronidase established.

The actual occurrence of substrates for hyaluronidase in the skin was also demonstrated (Claude, 1940; Chain and Duthie, 1940), and was later confirmed by chemical isolation of hyaluronic acid from that tissue (Meyer and Chaffee, 1941).

The finding that the hyaluronic acid of synovial fluid is probably identical with one of the main constituents of the ground substance of connective tissue (see the reviews by Meyer, 1947; and Duran-Reynals, 1942, 1950) brought new aspects to problems concerning the synovial polysaccharide. More particularly, studies on the structure and function of the synovial membrane (see Bauer *et al.*, 1940; Edlund, 1949; Gardner, 1950) as well as on the general chemical composition of normal synovial fluid (see Ropes *et al.*, 1939, 1940) led to the current view of the joint cavity as an enlarged tissue space, and to the belief that the hyaluronic acid of synovial fluid is formed by the same kind of cells in the peri-articular tissue as in connective tissue in general (see Asboe-Hansen, 1950). The study of the synovial polysaccharide should accordingly be able to provide certain information about the state of the connective tissue surrounding the joint, and possibly also of the mesenchymal tissue in general.

The results summarized above suggest the possibility that disturbances in the hyaluronic acid—hyaluronidase system might be factors of importance in the pathogenesis of joint disease, in particular the group called 'collagen diseases'. Further stimulus to the extensive study and discussion on this subject in recent years was the isolation of hyaluronic acid from group A and C streptococci by Kendall *et al.* (1937), followed by the demonstration of hyaluronidases in other strains of haemolytic streptococci (Meyer *et al.*, 1940, 1941). Since then numerous attempts have been made to explain the mechanism of rheumatic processes in terms of disturbances in the hyaluronic acid—hyaluronidase system (see reviews on the subject by Cohen, 1949; Ragan and Meyer, 1950; Stary and Tekman, 1951; Ragan, 1952).

In the present work interest has been concentrated on the changes in the synovial polysaccharide in joint diseases. Little has been written on this subject.

According to Ropes *et al.* (1947), qualitative changes from the normal occur in the 'mucin' of pathological joint fluids. In most fluids a lowered viscosity at unit concentration of mucin glucosamine was thus observed. This 'break-down' was obviously comparable only to

the initial stage of the enzymatic hydrolysis *in vitro*, since free N-acetylhexosamine could not be demonstrated in the fluids.

Associated with the decrease in viscosity there was a changed appearance of the mucin precipitate which occurs on adding acetic acid to the fluid. Thus, instead of the firm clot given by normal mucin, soft masses or flocculent precipitates in cloudy solutions or, in the case of high degrees of change, only a colloidal turbidity, were obtained with pathological fluids.

A decreased nitrogen/glucosamine ratio of the mucin usually paralleled the decrease in viscosity and the altered appearance of the precipitate in pathological fluids.

Ropes *et al.* found the tests of value in the differential diagnosis of joint disease. Thus mucin changes were essentially absent in both traumatic disorders and degenerative joint disease, but in fluids from rheumatoid arthritis or infectious arthritis of known aetiology marked alterations were usually present.

The changes observed were ascribed to a lower degree of polymerization of the mucin of pathological fluids. Whether this should be explained as resulting from a degradation process or an incomplete synthesis could not be decided. Active hyaluronidase could not be demonstrated in the fluids.

The intention of the present work, begun in 1947, was to supply information on the degree of polymerization of the synovial hyaluronic acid in normal and pathological fluids by means of determinations of viscosity and hyaluronic acid concentration.

For a quantitative approach to this problem determinations of mucin concentration were obviously unsatisfactory on account of its varying chemical composition. It was therefore necessary to elaborate a method for direct determination of the hyaluronic acid component in the fluids, and also to adapt the method to small amounts of test material in order to allow determinations on normal human fluid (chapter III). Further, comparison of the viscous behaviour of synovial fluids with that of hyaluronate solutions had to be made in order to examine whether the relationship between intrinsic viscosity and particle-size found by Blix and Snellman (1945) might be applicable to determination in naturally-occurring fluids (chapter IV). Comparisons of the rate of degradation and the shape of the viscosity reduction curve after addition of hyaluronidase to joint fluids and hyaluronate solutions were further included in the study, with the aim of elucidating the nature of the synovial polysaccharide. For this it was necessary to work out a method which allowed comparison of enzymatic activities in spite of varying degrees of polymerization of the substrate (chapter V).

By the methods worked out a series of normal and pathological synovial fluids was analysed. The first results obtained included the observation that in fluids of subacute and chronic polyarthritis the

hyaluronic acid was of lower mean polymerization than in fluids of traumatic arthritis or in normal fluids. (A short report on these results was given by Blix, 1948 A.)

Ragan and Meyer (1949) arrived at a similar conclusion in a study of rheumatoid arthritis fluids. They used a turbidimetric method (Meyer, 1947) for estimation of the hyaluronic acid content of the pathological fluids. By previous treatment of the samples with 0.01 unit of hyaluronidase the hyaluronic acid content of normal fluids, obtained by post mortem puncture, could also be determined.

The quotient between the log of the viscosity and the hyaluronic acid concentration was used as an index of the mean polymerization of the polysaccharide. The decrease from normal in this quotient was found roughly to parallel the activity of the disease. Ragan and Meyer ascribed the fluid changes in rheumatoid arthritis to an overproduction of depolymerized hyaluronic acid. They were unable to demonstrate active hyaluronidase in the fluids.

In chapters VII—IX the details of an extended study of normal and pathological joint fluids are given. A preliminary report of some of the results presented there has already appeared (Sundblad, 1950).

CHAPTER II

Materials and General Methods

Hyaluronate and chondroitin sulphate preparations. The pure polysaccharide preparations were placed at my disposal by Professor Gunnar Blix. They were all neutral Na-salts, isolated according to Blix and Snellman (1945), or, in most instances, essentially as described by Blix (1951 B). The latter procedure involved treatment with papain, dialysis, and removal of protein residues by the Sewag technique. In the isolation of hyaluronate from the umbilical cord, which was the usual source, special care was taken to remove as completely as possible all vessels in order to reduce the amount of sulphated polysaccharides often present in such preparations. Because of this, sulphur was essentially absent from the preparations (below 0.1 %). The analytical data for the hyaluronate preparations were essentially the same as given by Blix and Snellman, although the difference between the observed and theoretical contents of glucosamine was less marked. The average glucosamine content of 9 preparations as determined by the method of Blix (1948 B) was thus 40.0 %, compared with 44.6 % calculated for the sodium compound. The average N-content of these preparations was 3.30 % as compared with the calculated value of 3.49 %.

As substrate for the continuous control of the hyaluronidase used (see below) the same umbilical cord preparation was used throughout the main part of the investigation. This preparation had a relative viscosity of about 6 at 20° C. and at a concentration of 0.1 g. per 100 ml. in phosphate-NaCl (pH 7.0 and ionic strength 0.20). A 0.3 % solution of the standard preparation in 0.2 M-NaCl retained its viscosity and glucosamine content unchanged for at least several months if stored at — 18° C., and could thus conveniently be used in order to check the method of determining hyaluronic acid. Fresh dilutions with buffer were prepared from the stock solution about twice a month, and stored at + 4° C.

Some of the other preparations used were slightly more viscous, although the viscosities were always lower than reported for preparations made according to Hadidian and Pirie (1948) or Jeanloz and Forchielli (1950).

One of the chondroitin sulphate preparations was prepared from the cartilage of septum nasi of cattle, and the other from bovine articular

cartilage. The analytical data were essentially as given in the paper by Blix and Snellman, although the glucosamine values were somewhat higher.

The analytical values given show the preparations to be very pure. The absence of proteins and protein break-down products was further evidenced by negative ninhydrine tests.

Hyaluronidase. Preparation and determination of activity. The preparations ordinarily used were prepared from bull testes according to Madinaveitia and Quibell (1941). A suspension of hyaluronidase precipitated with 70 % saturated ammonium sulphate was used as stock solution. Of this suspension 10 ml. was dialysed free from salt, and then filtrated through a Seitz filter and made up to 50 ml. with phosphate-NaCl of pH 7.0. The activity of this solution did not change appreciably for at least two weeks if stored at + 4° C. A modification of the viscometric method designed by Madinaveitia and Quibell (1940) was used for the continuous control of the enzyme preparations used in the determination of hyaluronic acid (chapter III).

The relative viscosity of a 0.1 % solution of the standard hyaluronate in the ordinarily used phosphate-NaCl mixture (pH 7.0 and ionic strength 0.20) was measured at + 37° C. 0.1—0.2 ml. enzyme solution was then introduced into the viscometer, which contained 3 ml. substrate solution throughout. Rapid mixing was ensured by blowing the enzyme solution from a weighed pipette directly into the substrate, and then emptying the pipette by repeatedly sucking and blowing the mixture for about a minute. The viscosity was then determined at intervals of about 5 min., and the time taken to reduce it to half its initial value was determined by graphic interpolation.

The activity was given in terms of viscosity reducing units, VRU (McClean and Hale, 1941), per ml. test solution, the unit being defined as the amount of enzyme required to reduce the viscosity of 3 ml. 0.1 % standard hyaluronate, under the measuring conditions defined, to half its initial value in 20 min. The method of computing the results elaborated by Swyer and Emmens (1947) could be used with about the same accuracy for the comparative activity determinations, in spite of the fact that the substrate concentration was higher than that used by these authors. This procedure has the advantage that the reaction time is kept constant at 20 min. The reaction rate is in this case proportional to the log of the enzyme concentration, whereas the rate, determined by the half-viscosity value, is directly proportional to the enzyme concentration. The standard error of both procedures was found to be about ± 10 % as long as the standard hyaluronate was used.

The apparent activity of the same enzyme solution was, however, found to vary widely if different hyaluronates were used as substrate, other factors being equal (see chapter V). In order to allow comparison

with the data of other authors, parallel tests were made using a commercial hyaluronidase preparation ('Hyalas', Leo, Sweden), the activity of which was given as about 500 units per mg. This corresponds to about 1 300 VRU per mg., if assayed with the standard substrate as described above. The hyaluronidase solutions used in the routine determinations of hyaluronic acid (see chapter III) contained between 700 and 1 000 VRU per ml., corresponding to about 300—500 VRU per mg. protein.

Synovial fluids. The synovial fluids from horses were obtained by puncture of the astragalo-tibial joint as soon as possible after slaughter. The fluids from calves and rabbits were collected by means of sucking with a pipette after post mortem opening of the joints. Except for a few bursa fluids the pathological effusions were all obtained by aspiration of the knee joint, and had been sent for analysis from several different Swedish hospitals. The samples were handled under sterile conditions, and stored at — 18° C. if immediate analysis was not possible. Many of the fluids contained fibrin[1], which was removed by centrifugation before performing the tests. The supernatant was pipetted off, and as a rule diluted with buffer-NaCl before the viscosity determination. The determinations of hyaluronic acid and total protein were ordinarily performed on the sample which had been used for the viscosity determination. The changes in viscosity occurring on storage at + 37° C. were usually followed for about 20 hours. Because of this, and because the determination of hyaluronic acid involved incubation of the samples at + 37° C. for 48 hours, it was found necessary to perform all tests under essentially sterile conditions. Bacterial growth was further impeded by the addition of a small drop of toluene to the test samples.

Buffer solution. Unless otherwise stated, a salt-buffer mixture of pH 7.0 and ionic strength 0.20 (0.08 as phosphate and 0.12 as NaCl), was used as solvent for the polysaccharide preparations. The dilutions of the joint fluids and of the enzyme solutions were also made with this standard buffer.

Chemical methods. Hexosamine was determined by the method of Elson and Morgan (1933) as modified by Blix (1948 B); nitrogen by the micro-Kjeldahl method; and ester-bound sulphate gravimetrically, after hydrolysis with 2 N-HCl for about 14 hours at 100° C.

The method of determining hyaluronic acid in synovial fluids is described in chapter III.

The total protein content of naturally-occurring fluids was calculated directly from the estimated total nitrogen content. The non-protein

[1] Clot-formation was most marked in fluid from rheumatoid arthritis. The hexosamine/nitrogen ratio was determined in the centrifugates in 3 cases. The results indicated that only insignificant amounts of synovial polysaccharide were contained in the clots.

nitrogen was thus not taken into account. The error caused by this is usually negligible but may amount to about 10 % of the total value for normal fluids of low protein content. The hyaluronic acid nitrogen is insignificant, at most 2—3 % of the total, even in normal fluids.

The 'total protein' thus includes the protein component of the 'mucin', which should be borne in mind in comparing the results with values obtained after removal of the mucin by acetic acid precipitation. For normal fluids this difference will be considerable, since between 75 % (Ogston and Stanier, 1950) and 85 % (Ropes *et al.*, 1947) of the mucin of normal cattle fluids consists of protein. For most pathological fluids the mucin-fraction of the total protein will be of the order of 10—20 %.

Determination of viscosity. The viscosities were measured in a water-bath, usually at $37 \pm 0.1°$ C. η_{rel} indicates relative viscosity, i.e. the ratio of efflux times for solution and solvent, $\eta_{sp} = \eta_{rel} - 1$, specific viscosity. The intrinsic viscosity, i. e. the limiting value of the ratio $\frac{\eta_{sp}}{c}$ (c = concentration, given throughout in g. solute per 100 ml.) at zero concentration, is represented by the symbol $[\eta]$.

For routine measurements ordinary Ostwald viscometers (capillary length of about 10 cm.; times of outflow for water at 20° C. of about 15—45 sec.) were used. When only small quantities of test sample were available the determinations were made in viscometers of the semimicro type designed by Cannon and Fenske (1938). These required less than 0.5 ml. test fluid, compared with 3 ml. in the Ostwald viscometers, and had water values at 20° C. ranging from 7 to 130 sec.

Anomalous viscosity was studied in a horizontal capillary, which was connected with a manostat similar to that described by Ubbelohde (1937), and which could maintain constant pressures within the range 2—30 cm. water. The capillary was sealed at each end to graduated tubes of about 2 mm. bore, and was placed in a wider glass tube through which water of constant temperature could circulate. The fluid meniscus in the graduated tube was observed with the aid of a measuring microscope. The capillary used in most of the experiments had a length of about 10 cm. and a bore of 0.024 cm. The average rate of shear, β, was calculated as $\beta = \frac{8}{3} \cdot \frac{Q}{\pi r^3 t}$ sec.$^{-1}$, where Q is the volume of liquid flowing through a capillary of radius r in the time t.

For the quantitative treatment of the flow anomalies it was found convenient to relate the variations in apparent intrinsic viscosity to the shearing force applied rather than to the resultant rate of shear in the capillary. For this purpose the maximum pressure gradient, τ, was calculated as $\tau = \frac{P \cdot r}{2 \cdot l}$ in dynes per cm^2., where P is the pressure

difference across the capillary, r its radius, and l its length. By using the values of τ, determinations made in capillaries of different dimensions could be compared with satisfactory accuracy (see chapter IV). Disregarding slight deviations in density of the test solutions from that of the solvent, a constant, depending on the dimensions of the capillary and the average driving head, can be calculated for the Ostwald-type viscometers. The range of the values was for the ordinary Ostwald viscometers about 15—18, and for the viscometers according to Cannon and Fenske about 7—15.

STATISTICAL METHODS

Ordinary standard methods were used.

When comparing two means the method indicated by Fisher (1948) was used for small numbers of observations.

Regression lines were computed according to the method of least squares.

The rank correlation method (see e. g. Kendall, 1948) was used to test for independence, and to estimate the degree of correlation in some cases where the nature of the experimental data rendered treatment by ordinary methods less convenient. In other cases Fisher's method was employed.

Statistical values obtained were regarded as significant on the 1 % level.

The statistical errors of the analytical procedures are given in connexion with the descriptions of the methods employed. They were usually calculated as the errors of single determinations, σ_i, according to the formula (see Dahlberg, 1940) $\sigma_i = \pm \sqrt{\frac{\Sigma d^2}{2n}}$, where d is the difference between two observations, and n is the number of duplicate determinations.

CHAPTER III

Determination of Hyaluronic Acid

Until recently only indirect methods have been available for the assay of the synovial polysaccharide. These have usually been based upon determination of the nitrogen content of the hyaluronic acid—protein complex formed on dilution and acidification of the fluid.

The proportion of hyaluronic acid and protein in the 'mucin' thus obtained has been studied in particular by Ropes *et al.* (1947) under different experimental conditions. After reprecipitation from weak alkaline solutions these workers obtained products of constant composition from normal cattle synovia. The polysaccharide content was 15 % as calculated from hexosamine analyses, and the nitrogen/glucosamine ratio was 1.54. For mucin isolated from normal human fluid this ratio was 1.16, and for mucins of pathological fluids a variation between 0.49 and 1.69 was reported. A decrease in the nitrogen/glucosamine ratio from the normal of 1.5 was thus usually found in pathological fluids containing partly degraded hyaluronic acid. This is evidently in keeping with the fact that enzymatic degradation of the hyaluronic acid causes a progressive decrease in the amount of protein carried down with the polysaccharide on acidification of hyaluronate-serum mixtures, which is the basis of the turbidimetric assay of hyaluronidase (Kass and Seastone, 1944). The decrease in viscosity of hyaluronate, effected chemically by isolation methods, has also been shown to be accompanied by a decrease in ability to precipitate protein (Meyer, 1948).

Apparently the hyaluronic acid content of pathological fluids can only be approximately determined by means of mucin analyses, owing to the varying chemical composition. Apart from this the method is laborious, since the high protein content of such fluids necessitates repeated precipitations to remove the 'non-mucin' nitrogen.

Meyer (1947) has described a method of direct assay of hyaluronic acid in joint fluids, based upon determination of the stable colloidal turbidity obtained when partially degraded hyaluronic acid is precipitated by acetic acid in the presence of proteins. Evidently the method cannot be employed for analysis of normal synovial fluid which gives a fibrous mucin clot on acidification. This author applied the method

to such fluids by incubating them with 0.01 unit of hyaluronidase before analysis. This treatment, although sufficient to change the type of precipitate to a stable suspension, did not affect appreciably the turbidimetrically-determined hyaluronic acid content. Ragan and Meyer (1949) considered the accuracy of the method as satisfactory, but whether this would be true if the method were applied to pathological fluids containing hyaluronic acid of widely varying degrees of 'degradation' does not appear to be quite settled. The experience gained from the application of the method to hyaluronidase determination is, however, that under accurately standardized conditions the turbidity produced by hyaluronate of constant concentration is relatively independent of the source or method of preparation of the hyaluronate, especially if this is of low viscosity (Meyer, 1950; and others).

Attempts to determine hyaluronic acid in synovia by measuring the reducing substances released on enzymatic hydrolysis have been unsuccessful (Kulonen, 1951).

The method described below is based upon determinations of glucosamine, utilizing the specificity of the enzyme hyaluronidase for the estimation of that fraction of the total glucosamine content of the fluid which derives from the polysaccharide component.

EXPERIMENTAL

The non-hyaluronic acid fraction of the glucosamine of joint fluids belongs entirely to the carbohydrate component of the proteins. Removal of hyaluronic acid together with protein by means of precipitation with trichloroacetic acid and filtration, performed in a number of both normal and pathological fluids, was found to yield filtrates free from detectable amounts of hexosamine. Negative tests for free N-acetylglucosamine have been reported by several authors (Meyer, 1947; Ropes *et al.*, 1947), even in fluids showing marked changes in the polysaccharide. The protein-bound fraction of the hexosamine is relatively small in normal synovial fluids (usually below 20 %), but may exceed 50 % of the total in effusions of high protein content.

Early in this investigation the hyaluronic acid glucosamine was determined as the decrease in the total glucosamine content of the fluid brought about by removing the hyaluronic acid by means of complete enzymatic degradation and dialysis. Total hexosamine was first determined in 0.5—1 ml. test fluid. 1—2 ml. of the sample was then treated with testis hyaluronidase (100 VRU per ml. test sample was found to be a safe excess of enzyme) under simultaneous dialysis against 0.1 M-acetate buffer of pH 5.0, containing 0.3 M-NaCl. The dialysis was carried on at a temperature of 37° C. for 3 days with daily changing of the buffer. The entire contents of the dialysis bag

were then transferred to the glass bulb used in the hydrolysis required in the glucosamine determination (see below).

Control experiments showed that under these conditions practically all of the hyaluronic acid was eliminated during the first 24 hours, the process being complete in 48 hours. No further loss in glucosamine was effected by prolonged treatment with hyaluronidase, even if the concentration of enzyme was increased. Comparative tests using a highly purified enzyme (method of Hahn, 1943), and the partly purified hyaluronidase ordinarily used, gave identical results. As a further check, the loss of nitrogen brought about by prolonged hyaluronidase treatment and dialysis was determined in a sample of normal synovia, and was found to correspond well with the sum of the hyaluronic acid nitrogen and the non-protein nitrogen.

The procedure described above was, however, later abandoned for the following reasons. 1. Two duplicate determinations of glucosamine were required for each analysis of hyaluronic acid; 2. the amount of test fluid should preferably exceed 2 ml.; and 3. the proteins tended to precipitate in the dialysis bag, making the assay of protein glucosamine laborious and less accurate.

The procedure finally adopted is based on the fact that after complete degradation of the hyaluronic acid the proteins may be quantitatively precipitated, and then removed by filtration or centrifugation. The clear filtrate or supernatant is then analysed for glucosamine, using the method of Blix (1948 B) with slight modifications.

PROCEDURE

The test sample, usually 0.2—0.5 ml. joint fluid, was introduced into a 4-ml. measuring flask. Phosphate-NaCl mixture of pH 7.0, containing about 200 VRU hyaluronidase per ml., was added to a final volume of about 2 ml. After addition of a drop of toluene, the flask was placed in a thermostat at + 37° C. for 48 hours. The contents were then made up to the mark with 10 % trichloroacetic acid and thoroughly mixed. After standing for 20 minutes the mixture was filtered through a fine filter-paper (Munktell 00) using a cover-glass to prevent evaporation during the process. A 2-ml. aliquot of the clear filtrate was then transferred into a small Thunberg tube, marked at 5 ml., and 1 ml. 6 N-HCl added. The sealed tube was heated in an air thermostat at 100° C. for about 14 hours. The filtration or centrifugation usually needed when proteins are present could be omitted, as the hydrolysates were clear and usually colourless. The solutions could therefore be directly neutralized in the hydrolysis tubes. Early in the investigation this was done by careful titration with 5 N-NaOH, using a drop of 0.01 % methyl red as indicator. Although the methyl red

was found not to interfere with the Ehrlich colour-reaction, the titration was tedious in the narrow tubes, and the following procedure was therefore adopted.

The total hydrolysate was partly neutralized with somewhat less than the calculated amount of 5 N-NaOH, and then made up to the mark. Of this solution 1 ml. was carefully titrated with 0.25 N-NaOH from a microburette, using methyl red as indicator. From an identical microburette containing 1 N-NaOH, the amount required for neutralization was added to the remaining 4 ml. in the Thunberg tube, and the contents made up to the mark. This procedure was found to be rapid and accurate in serial determinations, and had the further advantage that overneutralization with strong alkali, which may easily cause destruction of hexosamine, was avoided.

Of the neutralized sample, 2 ml. was introduced into the Thunberg tube used for the acetylation, which was carried out according to Blix's directions. An acetylation time of 30 minutes was used throughout, which is adequate provided that the 2 ml. taken does not contain more than about 0.10 mg. glucosamine, corresponding to an extinction coefficient of about 0.35. After addition of the Ehrlich reagent the light-absorption was determined either in a Zeiss step photometer, filter S 53, or in a Beckman photometer at 530 mμ, using 10 mm. cuvettes throughout.

The standard procedure allows reliable determinations if the sample incubated with hyaluronidase contains more than about 0.3 mg. hyaluronic acid, provided that a Beckman photometer is used. The test sample should not contain more than about 1.3 mg. hyaluronic acid. The least amount of synovial fluid needed was thus found to be about 0.1—0.2 ml. for the range of hyaluronic acid concentrations encountered in this study.

If the available sample contained less than 0.4 mg. it was found better to use 4 ml. of the neutralized hydrolysate, in which case 4 ml. of the acetylacetone reagent was added and the reaction performed as usual. After cooling, 16 ml. alcohol and 2 ml. of the Ehrlich reagent were added. This modification proved to be as reliable as the 'standard procedure'.

The Thunberg tubes used for the hydrolysis had an outer diameter of about 10 mm., and had an additional mark at 8 ml., since it was sometimes convenient to use 5 ml. 2 N-HCl following the original directions for the method.

The hyaluronic acid concentration of the fluid was obtained by multiplying the observed polysaccharide-glucosamine content by the factor 2.36. This corresponds to a glucosamine content of 42.4 % calculated for the free acid, or 40.0 % for the Na-salt, which value represented the average found for protein-free hyaluronate preparations in this laboratory at the time of the present investigation.

NOTES ON THE PROCEDURE

The method was checked by analyses of mixtures of blood serum and hyaluronate solutions in varying proportions, the results being given in table 1.

Table 1. Determination of hyaluronic acid in mixtures of blood serum and hyaluronate solutions.

The results are expressed in terms of apparent titre of the hyaluronate solution tested, the average values being given. The calculated content was 0.170 g. per 100 ml., calculated as free acid. A human blood serum containing 8.4 g. total protein per 100 ml. was used.

Ml. serum added per ml. hyaluronate solution	No.	Hyaluronic acid found, g. per 100 ml.
0	9	0.167
0.25	6	0.162
0.50	6	0.172
0.75	6	0.166
Average of 27 determinations		0.167 ± 0.002
σ as per cent of M		± 5.4

A solution of the standard hyaluronate in phosphate-NaCl of pH 7.0 was used, which contained 0.170 g. per 100 ml. calculated as free acid. The digestion was carried out for 48 hours with about 200 VRU hyaluronidase per ml. test sample. The antihyaluronidase action of the serum was counteracted by the addition of a drop of toluene (see Adner, 1948). The hexosamine analyses were performed according to the modified procedure, employing direct neutralization of the hydrolysates after addition of a drop of diluted methyl red. The readings were made in the step photometer against blanks containing serum and hyaluronidase only, but otherwise treated as the test samples. These blanks did not contain measurable quantities of hexosamine.

Table 1 shows that the hyaluronic acid content of such mixtures could be estimated with reasonable accuracy. By employing a Beckman photometer for the readings, and by neutralizing the samples according to the 'standard procedure' given above, further accuracy could be achieved. The error of a single determination was found to be $\pm$ 4.2 % as computed from 25 consecutive duplicate determinations on hyaluronate solutions, or hyaluronate-serum mixtures performed as controls in connexion with the routine analyses of joint fluids.[1]

[1] Two controls with standard hyaluronate were usually included for every series of 10 analyses of joint fluids. This was found especially advisable when new preparations of acetylacetone or p-dimethylaminobenzaldehyde were tested.

The results of some further control experiments using solutions of pure hyaluronate will be briefly recorded. The estimated glucosamine content of such solutions was not significantly influenced by even prolonged treatment with strong hyaluronidase before analysis. These controls were done with regard to the reports by several authors (Meyer *et al.*, 1941; Hahn, 1945 A, B; Humphrey, 1946; Rogers, 1946) that the Elson and Morgan method (1934) for N-acetylhexosamine or modifications thereof may, if applied to enzymatic hydrolysates, yield values far in excess of the theoretical ones. Products are thus formed which give a red colour with the Ehrlich reagent even without preliminary treatment with alkali (Humphrey, 1946). However, these anomalies in the reaction with Ehrlich's reagent evidently do not interfere with the glucosamine determination *after complete acid hydrolysis*. The long hydrolysis time used also prevents any disturbing reaction between liberated non-nitrogen sugars and lysine (see Immers and Vasseur, 1950), since all sugars except hexosamine are completely destroyed during hydrolysis. The trichloroacetic acid did not disturb the reaction even in twice the ordinary concentrations.

The results of a series of analyses performed on a sample of pathological synovia under varying experimental conditions are given in table 2.

It is apparent that the accuracy of the determination was not appreciably affected by wide variation with regard to buffers, amount of hyaluronidase, or time of incubation, even if extra hyaluronate was added to the test sample before analysis. It should be noted that when analysing fluids of this high protein content (5.3 g. per 100 ml.) the test sample should preferably not exceed 0.3 ml.

Admixture of the samples with blood or haemoglobin derivatives did not interfere with the determination, the filtrates after precipitation with trichloroacetic acid usually being colourless. In some samples an orange colour was obtained with the Ehrlich reagent, presumably due to admixture of local anaesthetics containing p-aminobenzoic acid derivatives used at aspiration; the colour was identical with that given by minimal amounts of such compounds. When the readings were made in the step photometer blanks treated as the test samples, except for omitting the acetylation procedure, were always used. In the Beckman photometer these blanks had no measurable extinction at 530 mμ.

It should be noted that ordinary pipetting technique cannot be used in working with highly viscous joint fluids unless a trace of potent hyaluronidase is first added, in which case the viscosity drops almost instantaneously. Usually, however, the content of a weighed pipette was introduced into the incubation flask containing buffer and hyaluronidase, and the pipette emptied by sucking the mixture up and down repeatedly.

The necessity of working under essentially sterile conditions was emphasized in the introduction to this chapter. Although toluene

Table 2. Determination of hyaluronic acid in synovial fluid under different experimental conditions.

Equal volumes of test sample and a salt-buffer mixture containing the hyaluronidase were mixed and incubated at a temperature of + 37° C. The samples were then analysed for hyaluronic acid according to the 'standard procedure'. Different amounts of a hyaluronate solution containing 0.121 g. per 100 ml. were added to some samples before incubation as a further check. The joint fluid tested contained 5.3 g. total protein per 100 ml.

Units[1] of hyaluronidase added per ml. test sample	Buffer	Time of incubation, days	Mg. hyaluronic acid added per ml. test sample	Observed hyaluronic acid content of test sample, g. per 100 ml.
150	0.05 M-acetate	1	—	0.154
150	(pH 5.0) containing 0.15 M-NaCl	2	—	0.150
150		4	—	0.168
150		4	—	0.162
150	0.02 M-phosphate	1	—	0.152
150	(pH 7.0) containing 0.06 M-NaCl	2	—	0.156
150		4	—	0.157
350		4	—	0.164
350		4	—	0.168
120		1	0.72	0.156
120		2	0.72	0.150
120		4	0.72	0.146 [2]
200		4	2.00	0.167
200		4	2.00	0.154

Average of 14 determinations 0.157 ± 0.002
σ as per cent of M ± 4.6

[1] For definition of the unit (VRU), see page 120.
[2] The total values found were corrected for the hyaluronate added.

was also added before incubating at + 37° C. these precautions were insufficient to prevent bacterial growth in some samples, most of which were animal fluids. This was usually accompanied by marked increase in apparent hyaluronic acid content.

Unless secondary contamination with bacteria had occurred the fluids could be preserved at — 18° C. for several months without appreciable changes in hyaluronic acid concentration taking place. Thus on repeated analysis of 20 samples after a varying period of time (1—8 months, average 3 months) good agreement was found between the results of the first and the second test. The mean of the differences was + 2.3 mg. hyaluronic acid per 100 ml., with an ε (M) of ± 2.2 mg.

The experimental error for joint fluid analyses according to the 'standard procedure', calculated as the error of a single determination, σ_i, from a series of duplicate determinations, is evident from table 3.

Table 3. Accuracy of the assay of hyaluronic acid in joint fluids.

Two series of 25 duplicate analyses of joint fluids of all types were grouped according to the hyaluronic acid content of the fluid. σ_i is the error of a single determination.

Average hyaluronic acid concentration, M, mg. per 100 ml.	No.	σ_i	σ_i as per cent of M
69.8	25	3.1	4.4
177.0	25	7.5	4.3

The percentage error thus seemed to be of about the same magnitude both for high and low concentrations of hyaluronic acid. The actual experimental error should be considerably lower since with a few exceptions due to shortage of test material, duplicate determinations were always made.

The error of a single determination for the early modification employing enzymatic degradation and dialysis was ± 5.3 %, as computed from the combined σ_i values of 25 double determinations of 'total glucosamine' and 25 of 'protein-glucosamine'. In 7 fluids analysed according to this modification the average values obtained from 4 glucosamine determinations on each sample were compared with the results of 19 determinations according the the 'standard procedure'. On an average the latter values were 1.5 % higher, the ε (M) being ± 1.6 %, indicating good agreement between the two methods.

The finding by Rogers (1946) that only about 80 % of the reducing products of hyaluronate solutions are rendered dialysable by treatment with testis hyaluronidase could not be confirmed for the experimental conditions and enzyme preparations employed in the present study. In agreement, Meyer and Rapport (1952) state that no non-dialysable fractions are left following hydrolysis with testis hyaluronidase, a tetrasaccharide presumably being the predominant end product after prolonged hydrolysis.[1]

The method described allows direct determination of the glucosamine

[1] Ogston and Stanier (1952) have recently concluded that degradation of the synovial polysaccharide with testis hyaluronidase leaves a considerable fraction of the material in a polymerized state (molecular weight of the order of 10^6). This is to be expected for a degree of break-down such as that indicated by the values of η_{rel} given (1.75 after 24, and 1.51 after 48, hours, at a hyaluronic acid concentration of 1 mg. per ml.; the high end-value cannot be explained by the protein content of their starting material). Thus Hahn (1947) found that after degradation of a 1 % solution of pure hyaluronate to a relative viscosity of 1.5, only 2.9 % of the material was dialysable.

The cause of the incomplete break-down in the experiments of Ogston and Stanier is not obvious. Possibly too weak enzymes were used.

component of the hyaluronidase-sensitive polysaccharide in 0.1—0.2 ml. joint fluid with satisfactory accuracy. The corresponding hyaluronic acid concentration was calculated by multiplication with 2.36, assuming that the chemical composition of the synovial polysaccharide is identical with that of isolated hyaluronic acid. The validity of this assumption will be further discussed in chapter VI.

CHAPTER IV

Viscous Behaviour of Hyaluronate Solutions and Synovial Fluids

It has been shown conclusively that the viscosity of synovial fluid is chiefly due to its content of hyaluronic acid. Break-down of the polysaccharide with hyaluronidase or its removal by acetic acid precipitation reduce the viscosity to approximately that of water (Cajori and Pemberton, 1928; Ropes *et al.*, 1939, 1940; Meyer *et al.*, 1939, 1941), whereas protein-splitting enzymes leave the viscosity essentially unchanged (Ropes *et al.*, 1947).

On the other hand it has not been settled so far whether the differences in viscosity at unit concentration of polysaccharide observed between naturally-occurring fluids and solutions of isolated hyaluronate may simply be ascribed to differences in degree of polymerization of members of the same polymer-homologous series.

To determine this, the viscous behaviour of synovial fluid was studied under varying measuring conditions. In addition, comparisons with solutions of isolated hyaluronic acid were made in order to examine whether the relationship between intrinsic viscosity and particle-size found by Blix and Snellman (1945) for isolated hyaluronate might also be applicable to determinations in native fluids.

DETERMINATION OF THE SPECIFIC VISCOSITY OF THE NATURALLY-OCCURRING POLYSACCHARIDE

The contribution to the total viscosity of the proteins in the native fluids was determined by incubation of the fluids at 37° C. with potent hyaluronidase. The maximum decrease in viscosity obtained by this treatment is then the fraction due to the polysaccharide component. As seen from fig. 1, the viscosity is reduced to almost that of water on this treatment, the small difference being roughly proportional to the protein content of the fluid.

The magnitude of the correction for protein viscosity to be made when calculating the specific viscosity of the hyaluronic acid was determined in 4 samples of normal synovial fluid, and was found to vary between

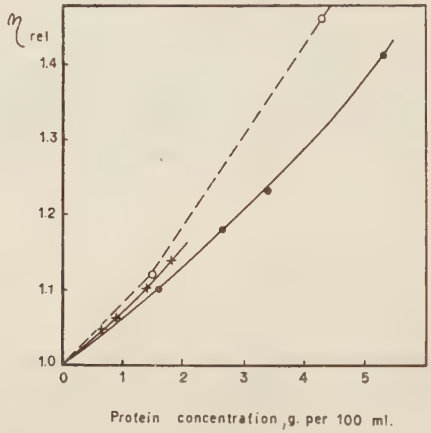

Fig. 1. Relative viscosity of synovial fluid as a function of protein concentration after complete degradation of the hyaluronic acid component.

× = dilutions of normal fluid from horse.
● = dilutions of a sample of pathological fluid.
– ⊙ – ○ – represents average values for the undiluted fluids of 4 samples of normal and 20 samples of pathological fluids.
Temperature 37° C. Dilutions were made with phosphate-NaCl of pH 7.0.

0.10 and 0.14, with an average value of 0.12. The average protein content of these fluids was 1.51 g. per 100 ml.

For 20 cases of pathological fluids of miscellaneous types, the average value was 0.46 ± 0.03 for an average protein content of 4.3 ± 0.3 g. per 100 ml. Since the determination was time-consuming and furthermore could not be performed in all cases due to shortage of test material, the averages of 0.46 for pathological and 0.12 for normal fluids were used throughout. The corrections to be applied for dilutions of the fluids were calculated on the assumption of a linear relationship between viscosity and concentration (see fig. 1). The error introduced by these approximations was estimated by calculation of intrinsic viscosities (see below) for 20 samples, using both the actually determined correction and the average value of 0.46. The error, σ_i, was then calculated from the differences, d, between the 'correct' and the 'approximate' values obtained, according to the formula

$$\sigma_i = \pm \sqrt{\frac{\sum d^2}{2n}}$$

The value of σ_i found for the 20 couples, expressed as per cent of the 'correct' average of intrinsic viscosity, was ± 0.53 %.

For normal fluids the approximation is negligible due to the high viscosity and low protein content.

RELATIONSHIP BETWEEN VISCOSITY AND CONCENTRATION. DETERMINATION OF INTRINSIC VISCOSITY

The results of a series of viscosity determinations at different concentrations of solute are given in fig. 2 for 6 hyaluronate preparations of varying degree of polymerization.

It appears that there is no linear relationship between viscosity and concentration except for hyaluronate preparations of very low viscosity in concentrations of up to about 0.05 % (see McClean and Hale, 1941; Swyer and Emmens, 1947). For more viscous preparations the steep rise of the viscosity/concentration curve even at relatively low concentrations is further evident, and is still more marked for the highly polymerized polysaccharide of normal synovial fluid.

It follows that the generally adopted basis for comparison of different hyaluronates by giving relative viscosities at an arbitrarily chosen concentration, usually 0.1 %, is less suitable in the case of highly viscous hyaluronate. The same is true for the method used by Ropes *et al.* (1947) for comparison of viscosities of synovial fluids and effusions. These authors found a direct proportionality between the log of the relative viscosity and the square root of the concentration of mucin-bound glucosamine in the fluids, and could thus, by means of interpolation from dilution curves, compare relative viscosities at a standard glucosamine concentration. Ragan and Meyer (1949) found a linear relationship between the log of the viscosity and the turbidimetrically determined content of hyaluronic acid in both normal and pathological synovia, the data thus being in conformity with the Arrhenius viscosity equation. Under the measuring conditions employed in this investigation, this formula was only approximately valid for highly viscous hyaluronate in a limited range of concentrations (up to about 0.05–0.1 %). For hyaluronic acid degraded with alkali according to Blix and Snellman (1945), and for chondroitin sulphuric acid, the relationship between log η_{rel} and concentration was, on the other hand, strictly linear up to concentrations of at least 0.25 %.

Huggins (1942) has shown that most formulae relating viscosity to concentration may be converted into the general formula

$$\frac{\eta_{sp}}{c} = \left(\frac{\eta_{sp}}{c}\right)_{c=0} + k' \left(\frac{\eta_{sp}}{c}\right)^2_{c=0} \cdot c + \text{terms in higher powers of } c \qquad (1)$$

or for dilute solutions

$$\frac{\eta_{sp}}{c} = [\eta] + k' [\eta]^2 \cdot c \qquad (2)$$

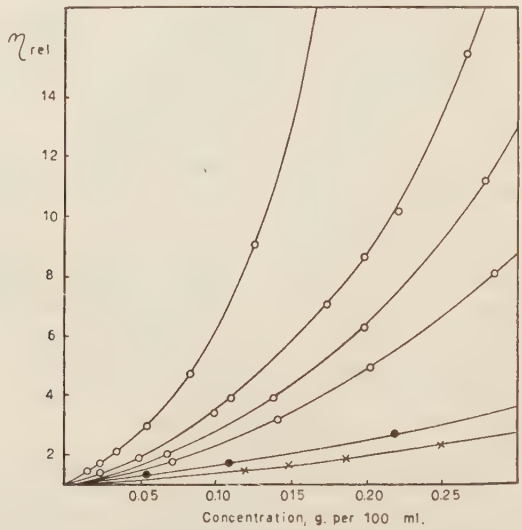

Fig. 2. Relative viscosity as a function of concentration for solutions containing hyaluronic acid of varying degrees of polymerization.

Solvent: phosphate-NaCl pf pH 7.0 and ionic strength 0.20. Viscosity determinations were made at 37° C.

○ = preparation from umbilical cord.
× = preparation from umbilical cord, degraded with alkali.
● = preparation from vitreous humour.

where $[\eta]$ = intrinsic viscosity; c = concentration of solute (in g. per 100 ml.). The constant, k', is characteristic of a given solute-solvent system. For solutions in a given solvent this constant is, however, the same for members of the same polymer-homologous series.

For the initial concentration dependence of the viscosity eq. (2) is equivalent to

$$\frac{\eta_{sp}}{c} = [\eta]\,(1 + k'\,\eta_{sp}) \tag{3}$$

The equation was first empirically derived in this form by Schulz and Blaschke (1941). These authors also tested its validity for polymers of marked polydispersity and found good agreement between the value of $[\eta]$ observed for an unfractionated preparation and the average of the intrinsic viscosities obtained for the fractions.

It is evident from eq. (3) that the value of $[\eta]$ both determines the intercept and influences the slope of the straight line obtained if $\frac{\eta_{sp}}{c}$ is plotted against η_{sp}. As seen from fig. 3 the data fit the equation both

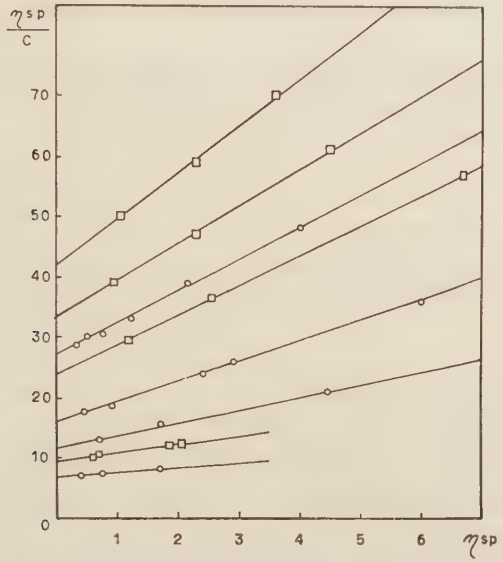

Fig. 3. $\frac{\eta_{sp}}{c}$ as a function of η_{sp}.

○ = hyaluronate preparations in phosphate-NaCl of pH 7.0, ionic strength 0.20.
□ = synovial fluids diluted with phosphate-NaCl.
Viscosity determinations were made at 37° C.

for hyaluronate solutions and dilutions of synovial fluids with buffer. The relationship was found to be approximately linear for η_{sp} up to about 15.

The constant, k', of eq. (3) was determined by calculation from dilution curves in a range of η_{sp} of about 0.3–10.

As is evident from table 4, the value of k' of eq. (3) was about the same for different hyaluronate preparations in spite of great variation in intrinsic viscosity. The relationship between viscosity and concentration was apparently different for chondroitin sulphate and for a preparation of hyaluronate from umbilical cord, degraded by alkali (see Blix and Snellman, 1945).

For hyaluronate from umbilical cord an average value of k' of 0.179 $\pm$ 0.004 was obtained from 10 dilution curves in all types of viscometers.

If k' is known, the intrinsic viscosity may be computed from the equation

$$[\eta] = \frac{\frac{\eta_{sp}}{c}}{1 + k' \eta_{sp}} \qquad (4)$$

Table 4. Intrinsic viscosity and k′ of eq. (3), calculated from dilution curves for solutions of hyaluronic and chondroitin sulphuric acid.

k' is the constant of the eq. $\frac{\eta_{sp}}{c} = [\eta] \cdot (1 + k'\eta_{sp})$ where $[\eta]$ is the intrinsic viscosity. Viscosity determinations were made at 37° C. Solvent: phosphate-NaCl, pH 7.0 and ionic strength 0.20.

Material	$[\eta]$	k'
Hyaluronic acid from umbilical cord	27.0	0.18
	16.6	0.17
	15.6	0.16
	13.0	0.18
	11.2	0.19
Hyaluronic acid from vitreous humour	6.70	0.13[1]
Hyaluronic acid, degraded with alkali	3.85	0.29
Chondroitin sulphuric acid	1.58	0.52

[1] Approximate value due to marked deviation from the straight line.

Table 5. Accuracy of intrinsic viscosity determinations in hyaluronate solutions.

Intrinsic viscosity of a hyaluronate preparation from umbilical cord, calculated from eq. (4) for $k' = 0.18$ in the range of $\eta_{sp} < 15$.

No.	Intrinsic viscosity $M \pm \varepsilon(M)$	σ	σ as per cent of M	Remarks
15	27.1 ± 0.1	0.43	1.6	Determinations made in viscometers of approximately the same dimensions.
21	26.8 ± 0.2	0.70	2.6	Determinations in all types of viscometer.

It is thus only necessary to determine the η_{rel} at an arbitrarily chosen low concentration of solute.

The validity of eq. (4), using the average value for k' of 0.18, for hyaluronate solutions is apparent from table 5.

Although eq. (4) was found to be approximately valid for viscosities of up to about 20, the determinations of intrinsic viscosity should preferably be made in the range of η_{sp} between 0.3 and 15 for an accuracy such as that of the data in table 5.

Table 6. Intrinsic viscosity and k′ of eq. (3), calculated from dilution curves of synovial fluids.

For explanation of signs and symbols, see table 4. The values of $[\eta]$ and k' given for the pathological fluids are the averages for the sub-groups classified according to intrinsic viscosity.

Material	No.	$[\eta]$	k'
Normal synovia			
Human	1	44.1	0.17
Horse	1	41.3	0.16
Calf	1	56.5	0.12 [1]
Pathological joint fluids	7	41.0	0.16
	7	24.6	0.17

Average of k' for 17 fluids 0.162 ± 0.005

[1] Approximate value due to marked deviation from the straight line.

In table 6 are given the results of determinations of the constants of eq. (3) for dilutions of synovial fluids with phosphate-NaCl (pH 7.0, ionic strength 0.20).

In spite of great variation in intrinsic viscosity (9.0–56.5) the relationship between viscosity and concentration appeared to be much the same in all types of synovial fluids, to judge from the values obtained for k'.

Using $k' = 0.16$[1] the validity of eq. (4) was tested by calculating the error of a single determination of intrinsic viscosity from series of double determinations on all types of joint fluids.

The values of each pair were obtained by viscosity determinations in the range of $\eta_{sp} < 15$ at different degrees of dilution, employing either the same viscometer or two viscometers of slightly different dimensions for the two determinations.

The results given in table 7 indicate that the procedure could be used with about the same accuracy for different types of synovial fluid, in spite of great variation in average degree of polymerization. The error of method for determinations in different viscometers, given in the table, is valid for the range of dimensions of the Ostwald viscometers used in the routine determinations of intrinsic viscosity in joint fluids (see chapter II).

[1] For the routine determinations of $[\eta]$ the values of $\frac{\eta_{sp}}{1 + k' \cdot \eta_{sp}}$ of eq. (4), corresponding to the observed values of η_{sp}, were conveniently taken from graphs. $[\eta]$ is then obtained by dividing by the concentration.

Table 7. Accuracy of intrinsic viscosity determinations in synovial fluids.

Intrinsic viscosities were calculated from eq. (4) for $k' = 0.16$ in the range of η_{sp} below 15. σ_i = error of a single determination as computed from series of double determinations at different degrees of dilution. Group I was classified according to intrinsic viscosity. $M\ [\eta]$ = average intrinsic viscosity.

Group	No. of fluids	$M\ [\eta]$	σ_i	σ_i as per cent of $M\ [\eta]$
I. Values of η_{sp} of each couple determined with the same viscometer	15 15	23.8 42.0	0.54 0.81	2.3 1.9
II. Values of η_{sp} of each pair determined in viscometers of different dimensions [1]	20	35.5	1.00	2.8

[1] Average difference in pressure gradient for the 20 couples $= 1.9 \pm 0.1$ dynes per cm^2.

Table 8. Determinations of intrinsic viscosity at high concentrations of solute.

Intrinsic viscosities, $[\eta]_1$ and $[\eta]_2$, were calculated according to equations (4) and (5) respectively from series of duplicate determinations of $\eta_{sp_1} < 15$ and $\eta_{sp_2} > 20$. σ_i = error of a single determination. $M\ [\eta]$ = average intrinsic viscosity.

Material	No.	Range of η_{sp_2}	Difference $[\eta]_1 - [\eta]_2$ $M \pm \varepsilon\ (M)$	σ_i	σ_i as per cent of $M\ [\eta]$ [1]
Hyaluronate solutions	15	20—80	-0.30 ± 0.22	0.64	3.1
Synovial fluids	15	21—87	$+0.25 \pm 0.41$	1.11	3.1

[1] Valid only for determinations of η_{sp1} and η_{sp2} of each couple in viscometers of approximately the same dimensions. For a series of 15 double determinations in a range of η_{sp2} of 24—279, using differently dimensioned viscometers, a value of $\sigma_i = \pm 6.8\%$ was obtained.

As seen from fig. 4, the relationship between viscosity and concentration is different when the viscosity is > 20.

The relationship between $\frac{\eta_{sp}}{c}$ and η_{sp} was, however, found to be approximately linear also in the range of $\eta_{sp} > 20$, for the hyaluronate preparations tested. In analogy with the conditions in the range of low viscosities, the slope of the lines is seen to be influenced by the intrinsic viscosity. The data given in fig. 4 were found to fit the equation

$$\frac{\eta_{sp}}{c} = [\eta]\ (2.0 + 0.11\ \eta_{sp}) \qquad (5)$$

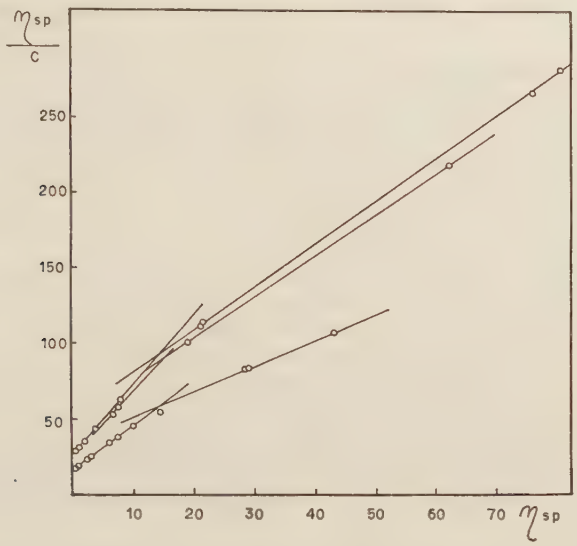

Fig. 4. $\frac{\eta_{sp}}{c}$ as a function of η_{sp} for hyaluronate solutions.

The constants given in eq. (5) are the averages obtained for the three experiments.

The validity of eq. (5) was tested for a wider range of data by comparing the intrinsic viscosities computed according to eq. (4), from determinations in the range of $\eta_{sp} < 15$, with the intrinsic viscosities obtained according to eq. (5), from determinations in the range of $\eta_{sp} > 20$.

The results given in table 8, representing data obtained with 8 different hyaluronate preparations and 15 synovial fluids of various types, indicate that the viscosity/concentration relationships in hyaluronate solutions and naturally-occurring fluids are similar also at high concentrations of solute.

As seen from the values of σ_i given in table 8, intrinsic viscosities could be computed according to eq. (5) with satisfactory accuracy from determinations in a wide range of viscosities.

INFLUENCE OF ELECTROLYTE CONCENTRATION, pH, AND TEMPERATURE

The viscosity of hyaluronate solutions is markedly influenced by variations in such factors as pH, temperature, and presence of neutral electrolytes. Thus Blix and Snellman (1945) found the relative viscosity

of a 0.15 % hyaluronate solution to be 9.45 in pure water but only 2.69 in 0.10 M-NaCl. The reversible reduction in viscosity produced by electrolyte addition to solutions of isolated hyaluronate or of naturally-occurring synovial polysaccharide has been studied by several workers (Madinaveitia and Quibell, 1940; Hadidian and Pirie, 1948; Ropes *et al.*, 1947; Ogston and Stanier, 1950). The effect has in both cases been shown to be most marked in the range of low salt concentrations. Above concentrations of about 0.1 M further electrolyte addition will thus only cause insignificant reduction in viscosity.

The effect of variations in hydrogen concentration upon the viscosity of polysaccharide and mucin solutions has been studied under varying experimental conditions by Ropes *et al.* (1947). It is evident from the results given by these authors that variations in pH, as well as in type or concentration of electrolytes of the degree which may be present *in vivo*, will be of only slight importance, and cannot to any extent explain the variations in viscosity encountered in synovial fluids. This is also illustrated by the data of table 9, where the influence of varying the solvent upon the observed intrinsic viscosity of hyaluronate is shown.

Table 9. Relation of intrinsic viscosity to ionic strength of solvent.

The averages of intrinsic viscosity, calculated according to eq. (4) for $k' = 0.18$, are given. Viscosity determinations were made at 37° C. for 3—4 different hyaluronate concentrations.

Material	Ionic strength of solvent	No.	Intrinsic viscosity 37° C.
Hyaluronate in phosphate-NaCl of pH 7.0	0.16	7	28.0
	0.20	8	27.0
	0.46—0.69	3	23.9
Hyaluronate in blood serum diluted with phosphate-NaCl of pH 7.0 and ionic strength 0.20 [1]		4	27.2

[1] Corrections were made for the serum viscosity.

Equation (4) (see page 137) was found to be approximately valid for the different solvents tested. A range of the values for k' of 0.16–0.19, with an average of 0.18, was thus calculated from the series of viscosity determinations recorded in table 9.

The slight variations in ionic strength arising from varying proportions of test sample and buffer in the routine analysis of the synovial fluids should accordingly have no significant influence upon the determination of intrinsic viscosity.

Table 10. Effect of temperature on the viscosity of hyaluronate solutions and synovial fluids.

The ratios between the specific viscosities, and the corresponding ratios between the intrinsic viscosities, are given. The series were classified according to relative viscosity, and the averages for the sub-groups are given. The subscripts denote the temperatures at which the determinations were made.

Material	No.	Range of $(\eta_{sp})_{20}$	Ratio $\frac{(\eta_{sp})_{37}}{(\eta_{sp})_{20}}$	Ratio $\frac{[\eta]_{37}}{[\eta]_{20}}$	
Hyaluronate solutions	11	5.3—30.0	0.830	0.917	0.917±0.007
	10	0.3— 5.2	0.888	0.916	
Synovial fluids	10	10.6—34.0	0.839	0.935	0.935±0.004
	9	1.8— 9.0	0.876	0.934	

Difference between the averages of the ratio $\frac{[\eta]_{37}}{[\eta]_{20}}$ for hyaluronate solutions and synovial fluids: 0.018±0.008 ($P<0.05$).

The influence of varying the temperature on the viscosity of hyaluronate solutions and synovial fluids is shown in table 10.

Confirming the findings of Ropes *et al.* (1947) the ratio of the viscosities at two different temperatures was found to vary with the viscosity of the test sample, being greatest in fluids of high viscosity. However, the shape of the viscosity/concentration curve was found to be the same at 20° C. and 37° C. for series of determinations on hyaluronate solutions at varying concentrations of solute. Thus the data at 20° C. were also found to satisfy eq. (3) (page 136), the value of k' being practically identical with that obtained for the same preparation at 37° C.

Accordingly the ratio of intrinsic viscosities at the two temperatures should be independent of the concentration of solute in contrast to the ratio of the specific viscosities, as is also evident from the results given in table 10. It is further seen that the average ratio of intrinsic viscosity is small both for hyaluronate solutions and for naturally-occurring fluids, although probably a slight difference between the two averages was present.

The findings seem notable in view of the interpretation of the temperature effect put forward by Ogston and Stanier (1951). These authors ascribed the decrease in viscosity brought about by a rise in temperature to a reduction in degree of aggregation of the particles. Regarding the greater tendency to molecular interaction at high concentrations of solute, a rise in temperature might then have been expected to alter the shape of the viscosity/concentration curve. There was, however, no evidence of this, as can be seen from the results in table 10.

ANOMALOUS VISCOSITY

On the basis of the streaming birefringence shown by hyaluronate solutions, in addition to the high viscosity even at low concentrations, a long-chain structure has been presumed for the molecule (Blix and Snellman, 1945). In analogy with the conditions observed for other asymmetrical macromolecules, hyaluronate solutions may thus be expected to show some type of structural viscosity. Lawrence *et al.* (1944), however, failed to demonstrate either streaming birefringence or anomalous viscosity (as measured in a viscometer of Couette type) in aqueous extracts of umbilical cord, in spite of the high viscosity of the solutions. In view of these findings they concluded that anisometric particles were probably not present in these preparations. Ferrari (1946) found on the other hand marked shear-dependence of the viscosity, both for solutions of mucin prepared by means of acetone precipitation of umbilical cord extracts, and for synovial fluids. Solutions of isolated hyaluronate showed the same behaviour but to a much lesser degree. He also studied the decrease in flow anomalies brought about by addition of electrolyte. Ropes *et al.* (1947) also stressed the non-Newtonian behaviour of the viscosity of synovial fluid, and stated that determinations should be made with viscometers with a bore sufficiently small to produce a rate of flow of 0.3 ml./min. or less. Ogston and Stanier (1950, 1951) studied the structural viscosity of normal bovine synovia with a Couette viscometer in a range of velocity gradients between 0.25 and 50 sec^{-1}. They found the flow anomaly to be a characteristic feature of the undegraded hyaluronic acid – protein complex assumed to be present in normal synovia. In contrast to the marked variation of viscosity with velocity gradient at high concentrations of solute, the viscosity of highly diluted solutions was found to be nearly independent of velocity gradient. After extrapolation of the viscosity/concentration ratios at zero concentration (i.e. the intrinsic viscosities) to zero and infinite velocity gradient respectively, a maximum difference of 0.1 between the logs of these values was thus indicated.

The findings were thus consistent with the idea that the particles are flexible chains for which no shear-dependence due to hydrodynamic orientation is to be expected since the particles are easily deformed (see Kuhn and Kuhn, 1945).

The viscous anomaly of synovia was accordingly ascribed to molecular interaction at higher concentrations of solute. The effect of the gradient was explained as a reduction in particle aggregation rather than as orientation of linear aggregates.

The purpose of the experiments described below was to study the dependence of the intrinsic viscosity on the rate of flow. A comparison of the shear-dependence between naturally-occurring fluids and solutions of isolated hyaluronate was further of interest as a possible source of information on the nature of the synovial polysaccharide.

EXPERIMENTAL

The anomalous viscosity of hyaluronate solutions and synovial fluids was studied in the horizontal capillary described in chapter II. The flow diagrams obtained are shown in fig. 5.

It is evident from the figure that hyaluronate of low mean polymerization does not show any flow anomalies under the measuring conditions employed. For preparations of high mean polymerization a shear-dependence was present although of only moderate degree. With the capillaries used it was not possible to obtain an accurate value for η_∞ (i.e. the viscosity at infinite rate of shear), since the curves showed no definite tendency to flatten out even at high gradients. This was especially marked in the viscosity curves obtained for synovial fluids where in addition only approximate values of η_0 could be obtained by extrapolation to zero rate of shear (see fig. 5). This was probably at least partly due to the relative inaccuracy of the determinations at pressures below about 2 cm. water, which was more marked for the joint fluids.

In the experiments with joint fluids, the flow times over a certain distance of the graduated tube were thus not quite constant at low pressures but tended to increase successively on consecutive determinations at constant pressure. The phe-

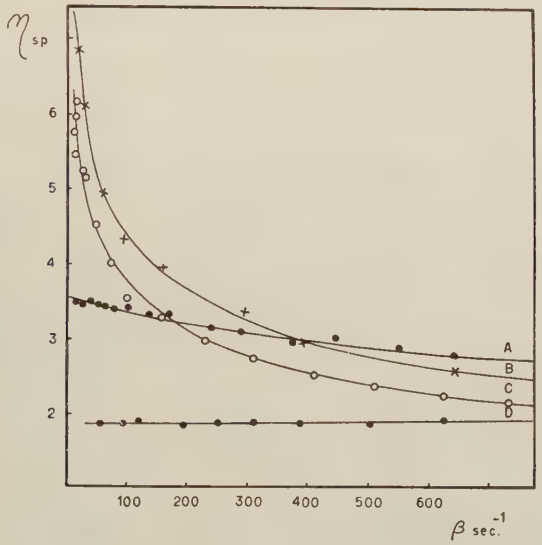

Fig. 5. Anomalous viscosity of synovial fluids and hyaluronate solutions.

β = average velocity gradient.
B, C = synovial fluid.
A, D = hyaluronate in standard buffer.

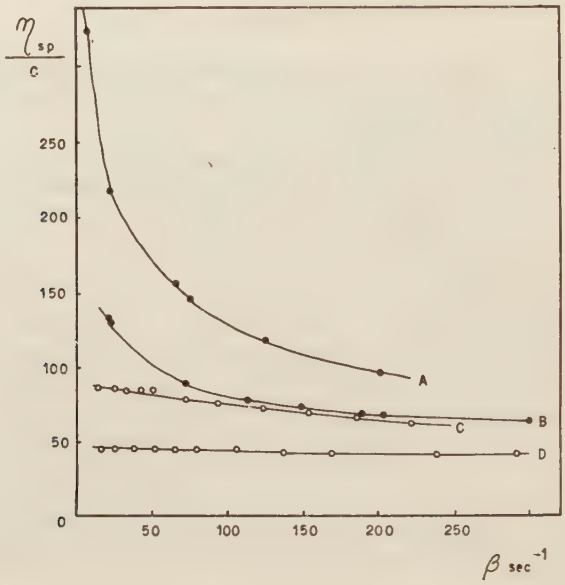

Fig. 6. Variation of $\frac{\eta sp}{c}$ with rate of shear.

β = average velocity gradient.
● = synovial fluid, diluted with standard buffer. A = 143 mg. hyaluronic acid/100 ml.; B = 43 mg./100 ml.
○ = hyaluronate in standard buffer. C = 150 mg./100 ml.; D = 75 mg./100 ml.

nomenon was not of measurable magnitude for pressures above 5 cm. in any case. The flow anomaly should apparently not be referred to the polysaccharide component of the joint fluids, to judge from the fact that solutions of isolated hyaluronate did not show this behaviour. Further, in a comparative test under identical conditions at a pressure of 2 cm. of water, the effect was found to be more marked for blood serum than for a sample of normal synovia of low protein content.

The viscous phenomenon may be analogous to the elasticity phenomenon demonstrated for blood serum by Blix (1933) under similar measuring conditions. Thus the reverse effect, i.e. an apparent decrease in viscosity, could be produced if the test fluid was first rapidly forced through the capillary in the opposite direction to the initial flow, and the time for the fluid meniscus to pass over a given distance of the graduated tube again measured.

It may be noted, however, that Ogston *et al.* (1950) have demonstrated a viscous-elastic behaviour for normal synovial fluid, which by these authors was ascribed to the polysaccharide component.[1]

The flow anomaly described was, however, not of a magnitude such as to influence appreciably the shape of the flow diagrams. Reproducible determinations could thus be made also in the range of pressures 1–3 cm. water by using the average values over a fixed distance in the graduated tube of the viscometer.

[1] This elastic behaviour of normal synovial fluid has recently been described in detail by Ogston and Stanier (1953).

Table 11. Anomalous viscosity of undiluted synovial fluid.

The sample tested contained 0.24 % hyaluronic acid. Viscosity determinations were made in the horizontal capillary at 37° C.

Pressure, cm. H_2O	$\eta_{rel.}$	Average velocity gradient, $sec.^{-1}$	$\frac{\eta_{sp}}{c}$	$[\eta]$ [1]
40	67	45.4	275	29.8
30	102	22.4	420	32.3
20	155	9.8	642	34.0
10	279	2.7	1 160	35.7

[1] Calculated according to eq. (5), valid only for determinations at constant pressures.

As seen from fig. 6 the shear-dependence of $\frac{\eta_{sp}}{c}$ increases rapidly with increasing concentrations of solute.

The data given in table 11 show clearly that values of relative viscosity for synovial fluids of high polysaccharide content have no significance unless the measuring conditions with regard to rate of flow are clearly defined.

Confirming Ogston and Stanier's findings (1951), the value of $\frac{\eta_{sp}}{c}$ at zero concentration was on the other hand found to be only slightly influenced by the velocity gradient, as indicated by the slight variation of the values of $[\eta]$ given in table 11. This is also illustrated by the good agreement between duplicate determinations of intrinsic viscosity, even if performed with different-sized capillaries (see tables 5 and 7, pages 138 and 140).

The formulae derived for the relationship between viscosity and concentration were found to hold for the whole range of viscometers of Ostwald, or modified Ostwald, type used in this study, provided that the different viscosity determinations of each dilution curve had been made with the same viscometer.[1] For the quantitative treatment of the flow anomalies it is accordingly expedient to relate the variations in apparent intrinsic viscosity to the shearing force applied (see chapter II, page 122) rather than to the resultant rate of shear. The latter will naturally vary for determinations at varying concentration of solute even if the same viscometer is used.

The results obtained in the horizontal capillary were in keeping with eq. (3), page 136, even for a wider range of pressure gradients (see fig. 7 and table 12).

[1] The average values given for k' of eq. (3) represent data obtained with viscometers of a range of pressure gradients between 7 and 18 dynes/cm^2.

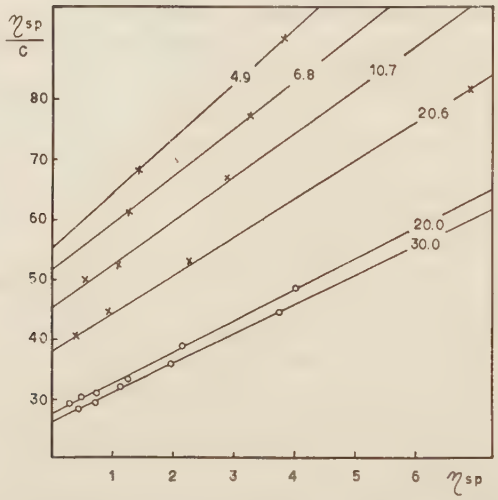

Fig. 7. $\frac{\eta_{sp}}{c}$ as a function of η_{sp}, applying different and constant pressures in the horizontal capillary.

○ = hyaluronic acid in standard buffer.
× = dilutions of a sample of synovial fluid.
The pressures in cm. water are given in the fig.

Table 12. Values of $[\eta]$ *and* k'*, calculated from dilution curves at different pressure gradients.*

k' is the constant of the eq. $[\eta] = \frac{\frac{\eta_{sp}}{c}}{1 + k'\eta_{sp}}$ (4).

Material	Pressure gradient, dynes per cm.^{2}	Intrinsic viscosity	k'
Hyaluronic acid dissolved in phosphate-NaCl of pH 7.0	18.0	26.4	0.18
	12.0	27.4	0.18
Joint fluid diluted with phosphate-NaCl of pH 7.0	16.2	38.0	0.16
	8.4	44.7	0.17
	5.3	50.5	0.16
	3.8	54.2	0.17

The relationship between viscosity and concentration for series of determinations at constant velocity gradient was, on the other hand, found to be different from that given by eq. (3).

For a rough comparison with the values of $[\eta]$, table 12, the values of log $\frac{\eta_{sp}}{c}$

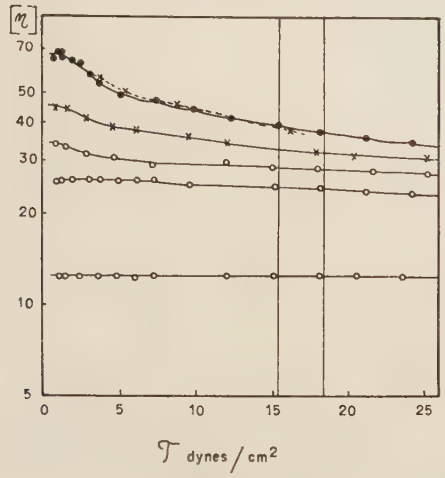

Fig. 8. Log $[\eta]$ as a function of pressure gradient.

● = normal synovial fluid from horse.

× = pathological synovial fluid. Dotted line gives the data for the sample of table 12. Each of the 4 values of $[\eta]$ was thus obtained by extrapolation to zero concentration from dilution curves at constant pressures.

○ = hyaluronate from umbilical cord.

The vertical lines indicate the range of pressure gradients, τ, of the Ostwald viscometers used in the routine determinations of intrinsic viscosity.

at constant rate of shear were approximately extrapolated to zero concentration for the sample of joint fluid of fig. 7. Using the difference between the values of log $[\eta]$ as a measure of the anomalous viscosity, the difference between the extreme values of 0.15 (see table 12) corresponded roughly to that obtained between the values of log $[\eta]$ at 75 and 450 sec.$^{-1}$ respectively. An extrapolation ventured to zero velocity gradient of the latter values of log $[\eta]$ would give a value of $[\eta]_0$ of the order of 65–70, roughly corresponding to the value for $[\eta]_0$ at zero pressure gradient (see fig. 8; dotted line represents the data for $[\eta]$ of table 12).

Assuming eq. (3) to be valid over the total range of pressures employed, the values of $[\eta]$ corresponding to those of $\frac{\eta_{sp}}{c}$ of the flow diagrams could be calculated from eq. (4).

The results given for hyaluronate solutions and synovial fluids in fig. 8 illustrate the variation of log $[\eta]$ thus obtained in the range of pressures 2–30 cm. water.

The validity of the procedure is evidenced in the figure by the approximate identity of the uninterrupted and the dotted line, the latter representing data obtained by extrapolation to zero concentrations from dilution curves at constant pressures.

The variation of $[\eta]$ is shown to be comparatively slight over a range

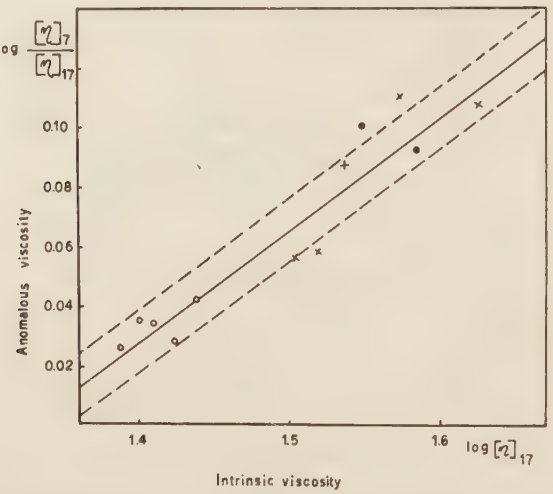

Fig. 9. Anomalous viscosity of hyaluronate solutions and synovial fluids.

As a measure of the anomalous viscosity the difference between the values of log $[\eta]$ at 7 and 17 dynes/cm.^{2}, taken from flow diagrams (see fig. 8), was used. Equation of regression line:

$$y = 0.382x - 0.506.$$

Dotted lines represent the vertical distance $\pm\sigma$ from the regression line. Correlation coefficient = 0.95.

of capillary dimensions such as that used in the routine determinations, although it is more marked for fluids of high intrinsic viscosity.

The relationship between the degree of viscous anomaly and intrinsic viscosity is illustrated by fig. 9. As a measure of the anomalous viscosity the difference between the values of log $[\eta]$ at pressure gradients 7 and 17 dynes/cm.^{2}, taken from flow diagrams such as those given in fig. 8, was used.[1]

[1] Ogston and Stanier (1952, 1953) have recently suggested that determinations of the (Couette) anomalous viscosity might be used in estimating the degree of degradation of the hyaluronic acid in pathological synovial fluids. The variation of viscosity with velocity gradient was found by these authors to be a more sensitive test of degradation than the Ostwald viscosity. It appears (see fig. 9) that the anomalous viscosity may equally well be measured in capillary viscometers, e.g. by determining intrinsic viscosities in two viscometers of different and fixed dimensions. The ratio between the two values of $[\eta]$ may obviously be directly calculated without measuring the concentration. It may well be that this ratio can be used as a sensitive criterion of the presence of undegraded polysaccharide. On the other hand the degree of flow anomaly cannot be used as a reliable measure of the mean polymerization in solutions of highly degraded hyaluronic acid (see fig. 9). In the case of marked polydispersity, which may be expected for the naturally-occurring polysaccharide (see Blix and Snellman, 1945), such solutions may still show pronounced anomalous viscosity arising from even small fractions of highly polymerized compounds.

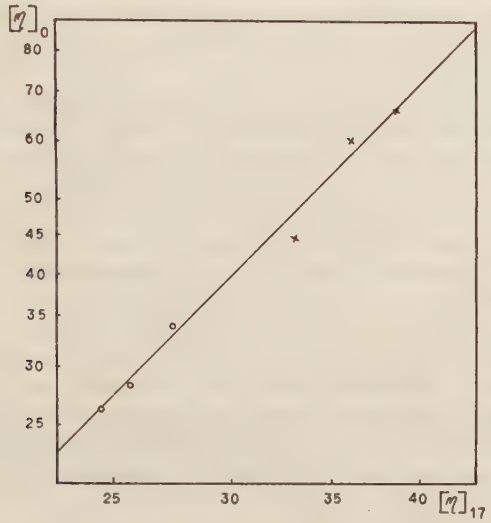

Fig. 10. Anomalous viscosity of hyaluronate solutions and synovial fluids.

The relation of the values of log $[\eta]$ at 17 dynes/cm.^{2} to those approximately extrapolated to zero gradient from flow diagrams (see fig. 8) is given.

× = synovial fluid.

○ = hyaluronate in standard buffer.

Equation of regression line: $\log y = 2.06 \log x - 1.45$.

To judge from the data of fig. 9, the much lower degree of anomalous viscosity observed for hyaluronate solutions compared with joint fluids might well be explained by the differences in mean polymerization. According to the regression equation obtained for the 12 samples, no measurable flow anomaly would be present over the range of gradients 7–17 dynes/cm.^{2} for hyaluronate of intrinsic viscosity levels below about 17. In agreement with this were the actual findings in tests using a hyaluronate preparation of $[\eta] = 12.4$.

Assuming the relationship between log $[\eta]$ and τ (fig. 8) to be linear in the range of gradients of about 7–17 dynes/cm.^{2}, the regression equation of fig. 9 may be utilized for comparison of intrinsic viscosities determined with different-sized capillaries. This procedure was tested for a series of duplicate determinations of $[\eta]$ in viscometers of the Ostwald type (average difference in pressure gradient $= 5.7 \pm 0.7$ dynes/cm.^{2}). After correcting the values of $[\eta]$ for the differences of gradient, a value of σ_i of ± 4.6 % was found for the 20 pairs, indicating that the procedure described above might be used with reasonable accuracy.[1]

[1] In a few instances the values of $[\eta]$ given in the following chapters were corrected to $\tau = 17$ dynes/cm.^{2}, since no determinations had been made in the range of gradients 15–18 ordinarily employed.

Although only approximate values for intrinsic viscosity at zero and infinite gradients could be arrived at with the equipment employed, it is evident from fig. 10 that the minimum values indicated for the difference between the logs of these values were considerably greater than those reported by Ogston and Stanier (1950, 1951). The maximum value of 0.1 given by these authors for normal bovine fluid corresponds roughly to the findings reported here for hyaluronate preparations of high mean polymerization. It appears that at least in part the discrepancy might be explained by the different measuring equipment. Ogston and Stanier extrapolated to infinite gradient from determinations in a cylinder viscometer over the range 0.25–50 $sec.^{-1}$, which may not be correct in the light of the observation made here using the capillary viscometer that accurate values of $[\eta]_\infty$ cannot be arrived at even for gradients of more than 1000 sec^{-1}. Similar findings have recently been reported for a synthetic polymer of probably much the same particle-size as naturally-occurring hyaluronic acid (Alexander and Hitch, 1952; see discussion in chapter VI).

CHAPTER V

Relative Degradation Rates with Hyaluronidase using Hyaluronate Solutions or Synovial Fluids as Substrate

In confirming the findings of several authors (Meyer, 1947, 1950; Lundquist, 1949; Dorfmann, 1950), the apparent activity of hyaluronidase assayed viscometrically (see chapter II, page 120) was found to vary widely if different hyaluronates were used as substrate, other factors being equal.

Since the object of the experiments described in this chapter was the comparison of reaction rates at fixed enzyme concentration between hyaluronate solutions and naturally-occurring synovial fluids as substrate, it was necessary to study the nature of this substrate-dependence of the viscometric hyaluronidase assay.

With solutions of isolated hyaluronates as substrate, variations in apparent activity arise from two different sources, lack of purity of the hyaluronate preparations, and varying degree of polymerization of the hyaluronate.

The influence of the second factor will be dealt with here, since for hyaluronate preparations of the purity indicated by the analysis data given in chapter II, page 119, the lack of reproducibility of the assay with different hyaluronates was found to be mainly due to the varying viscosity of the preparations. The methods of purifying the hyaluronate preparations used (see chapter II, page 119) evidently removed to a high degree impurities with inhibitory action on hyaluronidase (see Meyer, 1947; Meyer and Rapport, 1952). The essential factor was probably that the sulphated mucopolysaccharides, which may act as inhibitors of a presumably competitive nature (McClean, 1942; Meyer, 1947; Hadidian and Pirie, 1948), had been removed to a satisfactory extent. This was evidenced by low sulphur contents (below 0.1 %) and also by the fact that, if the degradation rate was determined as described below, practically identical values were obtained in comparative tests with preparations from vitreous humour and umbilical cord. Sulphate-containing impurities with inhibitory action are otherwise usually present in preparations from umbilical cord (Meyer, 1950).

References to the extensive literature on hyaluronidase assay and related subjects can be found in a recent review by Meyer and Rapport (1952), in which the kinetics and limitations of both viscometric and other physico-chemical as well as biological methods are discussed in detail on the basis of recent work in this field. The earlier literature will therefore be referred to only superficially.

DEGRADATION RATES WITH HYALURONATE SOLUTIONS

The influence of variations in mean polymerization of the substrate upon the viscometrically determined enzyme activity is illustrated by fig. 11.

The figure shows the results obtained for the preparations of highest and lowest viscosity out of 4 samples from umbilical cord tested at constant enzyme concentration. The preparations were of about the same degree of purity, as judged from the analytical data. The figure also illustrates the great influence upon the apparent activity of the enzyme tested, which is effected by an even comparatively slight fall in substrate viscosity, as it often occurs spontaneously in hyaluronate solutions on standing. By means of partial degradation with a trace of hyaluronidase before the determination an eightfold decrease in apparent activity, in terms of viscosity reducing units, was thus achieved for preparation $A–A_2$, table 13 (page 158). Variations of this magnitude have also been reported in comparative tests using hyaluronates of varying mean polymerization (Lundquist, 1949; Meyer, 1950), but so far no satisfactory method of eliminating errors of this type, inherent in the viscometric assay, has been found.

Before entering into the details of the methods worked out, it is necessary to discuss briefly the kinetics of the viscosity reduction.

Several workers have attempted to calculate reaction constants under the assumption that the reaction follows the kinetics of a monomolecular reaction (Werthessen *et al.*, 1945; Swyer and Emmens, 1947; Dorfmann, 1948; Lundquist, 1949). The procedures employed have, however, usually been found to apply only for narrow ranges of substrate viscosity.

Rapport *et al.* (1950) have pointed out that most kinetic treatments involve the probably erroneous assumption that the substrate fractions, already attacked by the enzyme, do not contribute to the total viscosity.

On analysis of the products of enzymatic hydrolysis, Meyer and Rapport (1952) thus found the results consistent with the theory that testicular hyaluronidase in fact attacks the substrate close to the middle of the polymer chain. Low-molecular fractions were not formed in the inital stages of the reaction.

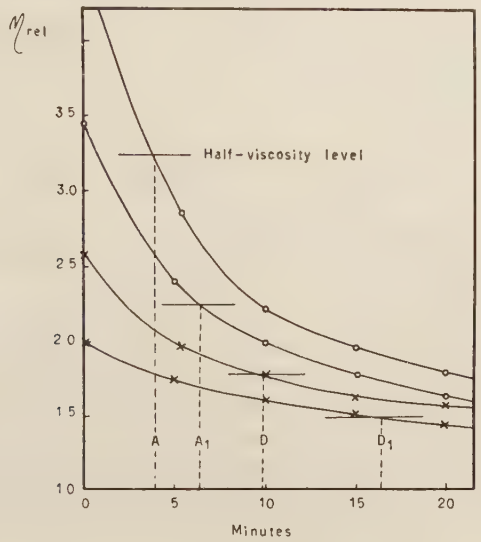

Fig. 11. Relation of the degradation rate, measured as the half-viscosity time, to the initial viscosity of the substrate.

0.2 ml. hyaluronidase solution and 3 ml. 0.1% hyaluronate in standard buffer were used. A_1 and D_1 are solutions of the two preparations after previous treatment with a trace of hyaluronidase. The initial viscosity was calculated from eq. (4), page 137, by substituting the value for concentration corrected for the enzyme addition.

Hultin (1948) has concluded that in the enzymatic breakdown of hyaluronate all (or almost all) linkages between the basic molecules are broken with equal ease. This author tried to apply a mathematically deduced equation for the decomposition of polymeric homologous substances (Hultin, 1946) to the experimental data published by Madinaveitia and Quibell (1940) and Swyer and Emmens (1947), but found no regular conformity.

Hultin could not decide whether the discrepancies were due to deviations from the Staudinger equation, upon which his formula was founded, or to some other factor.

EXPERIMENTAL

Assuming the enzymatic decomposition of hyaluronate to be a first-order reaction, the velocity constant, k, is given by the formula

$$k = \frac{1}{t} ln \frac{S_0}{S_t} \qquad \begin{matrix} S_0 = \text{initial substrate concentration} \\ S_t = \text{substrate concentration at time } t \end{matrix} \qquad (6)$$

On the assumption that the split products do not contribute to the total viscosity to any significant extent, the change in substrate concentration may be calculated from eq. (4), page 137. Equation (6) may then be written

$$k = \frac{1}{t} ln \frac{\eta_{sp_0}(1 + k' \cdot \eta_{sp_t})}{\eta_{sp_t}(1 + k' \cdot \eta_{sp_0})} \qquad \begin{matrix} \eta_{sp_0} = \text{initial specific viscosity} \\ \eta_{sp_t} = \text{specific viscosity at time } t \end{matrix} \tag{7}$$

Equation (7) was tested for the data given in fig. 11, but was found to apply only approximately within a narrow range of substrate viscosity, and could not be utilized for practical purposes.

The experimental data were therefore treated on the assumption that the linkages in the polymer chains are attacked at random with a successive reduction in average molecular weight of the substrate. The intrinsic viscosity, calculated according to equation (4), was further assumed to be directly proportional to the average molecular weight.

If the intrinsic viscosities at the beginning of the experiment, after t minutes, and at complete breakdown, are denoted as $[\eta]_0$, $[\eta]_t$, and $[\eta]_\infty$ respectively, the degradation rate constant, K, will be (af Ekenstam, 1936)

$$K = \frac{1}{t} ln \frac{1 - \frac{[\eta]_\infty}{[\eta]_0}}{1 - \frac{[\eta]_\infty}{[\eta]_t}} \tag{8}$$

Eq. (8) may be expanded into series as

$$K = \frac{[\eta]_\infty}{t} \cdot \frac{[\eta]_0 - [\eta]_t}{[\eta]_0 \cdot [\eta]_t} + \frac{[\eta]_\infty^2}{t \cdot 2} \cdot \frac{[\eta]_0^2 - [\eta]_t^2}{[\eta]_0^2 \cdot [\eta]_t^2} + \cdots \tag{9}$$

When $[\eta]_0$ and $[\eta]_t$ are large compared with $[\eta]_\infty$ the higher terms may be neglected. The formula may then be simplified to

$$K = \frac{[\eta]_\infty}{t} \cdot \frac{[\eta]_0 - [\eta]_t}{[\eta]_0 \cdot [\eta]_t} \tag{10}$$

which may be written

$$K = \frac{[\eta]_\infty}{t} \left[\frac{1}{[\eta]_t} - \frac{1}{[\eta]_0} \right] \tag{11}$$

Since the value of $[\eta]_\infty$ may be regarded as the same for the same enzyme/substrate system, eq. (11) may be written

$$K_1 = \frac{1}{t} \left[\frac{1}{[\eta]_t} - \frac{1}{[\eta]_0} \right] \tag{12}$$

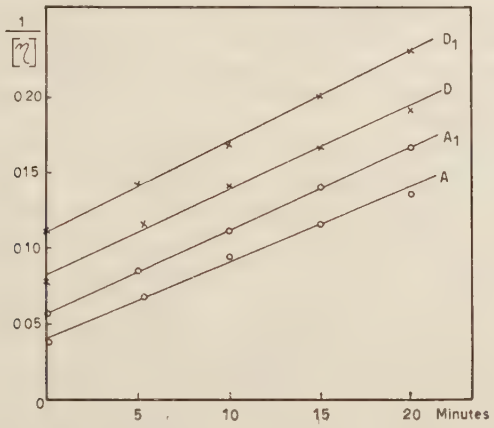

Fig. 12. Relationship between $\frac{1}{[\eta]}$ and reaction time on enzymatic degradation of hyaluronate of varying degree of polymerization.

For explanation see caption of fig. 11.

The degradation rate constant calculated according to eq. (12) should decrease as the reaction proceeds owing to the constant changes in the substrate. The decrease in number of available bonds might, however, be expected to be comparatively slight even for a marked reduction in relative viscosity of the substrate. Using a ultramicromethod for reducing sugar, Rapport *et al.* (1950) thus found that the number of bonds opened on treating hyaluronate with 1 turbidimetric unit for 30 minutes represented at most 2 % of the total hydrolysable bonds.

Equation (12) was tested for the experimental data given in fig. 11 by substituting the values of η_{rel} with the corresponding intrinsic viscosities according to eq. (4), page 137. Plotting $\frac{1}{[\eta]}$ against the reaction time, approximately straight lines were then obtained for the test period of 20 min., as can be seen from fig. 12.

It is evident from eq. (12) that the slope of the line will be equal to the degradation rate constant, K_1, and will thus be a measure of the enzyme activity.

In order to test whether the degradation rate can be directly calculated from viscosity determinations over a time interval chosen at random, the agreement of the experimental data with equation (13) was tested.

$$K_2 = \frac{10^3}{t_2 - t_1}\left[\frac{1}{[\eta]_{t_2}} - \frac{1}{[\eta]_{t_1}}\right] \text{ in } 'VU' \qquad (13)$$

where t_1 and t_2 indicate reaction times in min. VU = 'viscosity units'.

Table 13. Average degradation rate in 'viscosity units' (VU) for break-down of hyaluronate of varying mean polymerization with a fixed amount of hyaluronidase.

For explanation of the unit VU see text. The degradation rates given are the averages of 3 determinations over a time interval of about 10 min. each within the total time of 25 minutes.

Hyaluronate preparation		Intrinsic viscosity	Degradation rate
I Undegraded	A	27.0	6.25
	B	24.3	7.10
	C	14.7	6.65
	D	13.0	5.55
	Average	19.7	6.38
II Partly degraded	A_1	18.0	5.82
	B_1	16.2	6.10
	C_1	12.3	5.26
	D_1	8.9	6.28
	A_2	5.5	6.30
	Average	12.2	5.95

Table 14. Changes in degradation rate between 0 and 20 minutes.

The averages for the two series of experiments of table 13 are given.

Substrate	No.	Average degradation rate over time interval VU		
		0—10 min.	5—15 min.	10—20 min.
I. Undegraded hyaluronate	4	6.80	6.48	5.90
II. Partly degraded hyaluronate	5	5.92	5.90	6.04

The degradation rates for the 4 hyaluronate preparations tested, calculated according to eq. (13), are given in table 13 as the averages of 3 determinations over a time interval of about 10 min. (0–10, 5–15, and 10–20 min.).

Apparently the reaction rate, measured in these 'viscosity units,' was much the same for all the preparations tested in spite of the wide variation in mean polymerization. If, on the other hand, the rate was measured in VRU (viscosity reducing units, see page 120), a variation between 0.7 and 5.5 was present in the series of table 13.

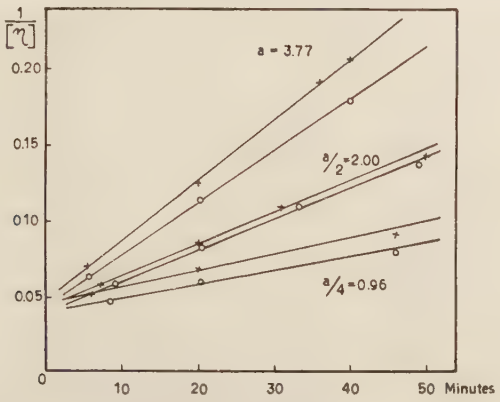

Fig. 13. Relation of degradation rate to enzyme and substrate concentrations. Hyaluronate in standard buffer: ○ = 0.1 g. per 100 ml. × = 0.05 g. per 100 ml. The average rates, in VU, are given in the fig. for the 3 enzyme dilutions tested. Average acitivity of 'a' (computed from 9 determinations with 3 hyaluronate preparations) = 3.85 VU. One of the preparations was from vitreous humour ($[\eta] = 6.7$), the others from umbilical cord ($[\eta] = 11.2–27.0$).

In table 14 are illustrated the changes in depolymerization rate during the progress of the reaction for the two series of experiments of table 13.

The table shows that the initial rates, i.e. between 0 and 10 min., were on an average somewhat higher in the experiments of series I, whereas the degradation rate was essentially constant over the test period of 20 min. if the hyaluronate solutions had previously been subjected to slow enzymatic depolymerization (series II). The difference can probably be ascribed to the fact that the preliminary treatment lessened the influence of polydispersity by eliminating the longest molecules, which ordinarily account for a comparatively large fraction of the initial viscosity even if present in small numbers.

Evidently the initial stage should be avoided when determining the degradation rate in this way. This is also advantageous in order to avoid calculation or determination of the initial viscosity of the substrate after dilution due to addition of the enzyme solution. Furthermore, errors due to the inability of enzyme and substrate immediately to mix are evidently eliminated.

The results indicate that the enzyme activity may be estimated with satisfactory accuracy from the degradation rate as computed from two viscosity determinations over a time interval of about 10 min., even if different substrates are used.

In the experiment illustrated in fig. 13 the rates were found to be practically constant for at least 40 min., and directly proportional to

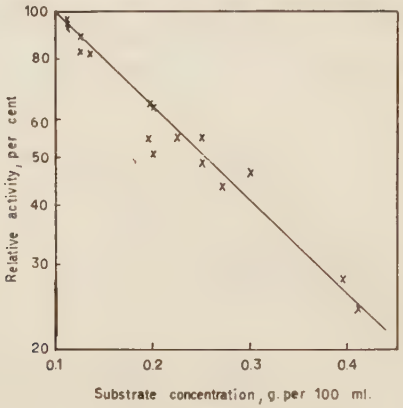

Fig. 14. Relation of degradation rate to substrate concentration.

All degradation rates were calculated relative to those obtained at a substrate concentration of 0.1 g. per 100 ml. under identical conditions. The data given represent results obtained with 4 different hyaluronate preparations tested at various enzyme concentrations in the range 2–8 VU (at hyaluronate concentration 0.1 g. per 100 ml.). Equation of regression line: $\log y = 2.20 - 2.0\,x$

Estimated mean deviation from the line $= \pm 0.036$.

the enzyme concentration. The influence of substrate concentration upon the degradation rate is seen to be comparatively small in the range of concentrations tested.

As a basis of comparison the degradation rate at a substrate concentration of 0.1 g. per 100 ml. was chosen, and used as a measure of the enzyme activity.

At lower concentrations the determination was often inaccurate due to short flow times.

The relation of the apparent activity, as measured by the degradation rate according to eq. (13), to the substrate concentration in the range above 0.1 g. per 100 ml. is shown in fig. 14.

The empirical relationship found between the log of the apparent activity and the hyaluronate concentration is seen to hold with reasonable accuracy in spite of highly varying experimental conditions.

By means of the diagram of fig. 14, activities obtained with different substrate concentrations could thus be compared.

Finally, the experiments shown in fig. 15 illustrate the influence of varied salt concentrations upon the degradation rate measured in VU.

Thus the ionic strength should be held essentially constant, whereas the proportion of NaCl and phosphate at constant ionic strength may be varied without appreciably affecting the result to judge from a few experiments performed.

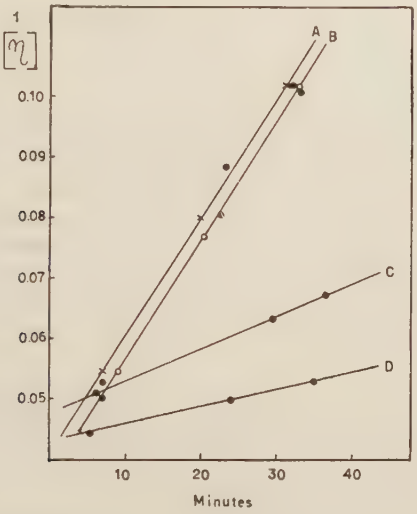

Fig. 15. Influence of salt concentration on the degradation rate at constant enzyme concentration.

Substrate: Hyaluronate in phosphate-NaCl mixtures of pH 7.0 and varying ionic strength.
A = 0.05 % hyaluronate. × = ionic strength 0.20
● = ionic strength 0.16
B = 0.1 % hyaluronate. ○ = ionic strength 0.20
● = ionic strength 0.16
C = 0.1 % hyaluronate. Ionic strength 0.46.
D = 0.05 % hyaluronate. Ionic strength 0.69.

DETERMINATION OF HYALURONIDASE ACTIVITY WITH SUBSTRATES OF VARYING DEGREE OF POLYMERIZATION

Based upon the results given above, the following standard procedure for the viscometric hyaluronidase assay was adopted in order to eliminate discrepancies due to variations in mean polymerization of the substrate.

Substrate and *enzyme* were mixed to a final hyaluronic acid concentration of 0.1 g. per 100 ml., e.g. by adding 1 ml. enzyme solution to 2 ml. of a 0.159 % solution of sodium hyaluronate in phosphate-NaCl of pH 7.0 and ionic strength 0.20.

A stock solution of hyaluronate in 0.2 M-NaCl was used and mixed with phosphate of ionic strength 0.20 in the proportion 3 : 2.

Two different dilutions of hyaluronidase in the standard mixture or 0.2 M-NaCl were as a rule used. If the initial determination of viscosity

was to be made within 5 minutes, the solutions were previously warmed to 37° C. 3 ml. of the substrate-enzyme mixture was then introduced into the viscometer.

Viscosity determinations were ordinarily made at about 5, 10, 15, and 20 minutes. The time should be exactly determined by adding the half values of the flow times. It was found preferable to use Ostwald viscometers with flow times for the solvent at 37° C. of about 15 (10–20) sec., and to avoid determinations in the range of viscosities above 10 in order to minimize the changes in viscosity during the individual determination of the flow time. With low flow times the advantage was further gained that the test could easily be applied to serial determinations. Thus parallel determinations in 6 viscometers could be performed in about 30 minutes.

The enzyme activity, A, was measured by the degradation rate, which was calculated as the average of two determinations over a time interval of about 10 min. (5–15 and 10–20 min., respectively) according to eq. (14), derived from eq. (13), page 157, putting $c = 0.1$.

Eq. (13) will then transform into

$$A = \frac{10^2}{t_2 - t_1}\left[\frac{1}{(\eta_{sp})_{t_2}} - \frac{1}{(\eta_{sp})_{t_1}}\right] VU \qquad (14)$$

t_1 and t_2 = reaction times in minutes.
VU = viscosity units.
Hyaluronic acid concentration 0.1 g. per 100 ml.
Range of specific viscosities at 37° C. $(\eta_{sp})_{t_1} < 10$, $(\eta_{sp})_{t_2} > 0.3$

Since the η_{sp} at 37° C. of a 0.1 % solution of the standard hyaluronate was about 5, it follows that for this preparation the unit VRU, as defined on page 120, will be approximately equal to the unit VU, since reduction of the viscosity by half will require

$$\frac{10^2}{20}\left[\frac{1}{2.5} - \frac{1}{5}\right] = 1\ VU$$

Thus 1 VU may also be defined as the amount of enzyme required to reduce the η_{sp} of 3 ml. 0.1 % solution of hyaluronic acid from 5 to 2.5 in 20 min. Using 2 ml. 0.15 % hyaluronate and 1 ml. enzyme solution, the titre in VU per ml. will be directly obtained from eq. (14).

NOTES ON THE PROCESS

The accuracy of the above procedure over a wide range of enzyme concentrations is illustrated by the data of table 15.

The variation in degradation rate in the experiments of table 15 corresponded to half viscosity times between 1 and 120 min. for the hyaluronate preparations used.

Table 15. Relation of degradation rate to enzyme concentration.

Enzyme solutions and hyaluronate in standard buffer were mixed to a final substrate concentration of 0.1 %. The dilutions of the hyaluronidase solution tested were made with 0.2 M-NaCl. The values in the first column give the enzyme concentrations expressed as ml. of the undiluted enzyme solution present in 3 ml. substrate-enzyme mixture. With the hyaluronate used 1.09 VU were required to reduce the viscosity of the mixture by half. The degradation rate was calculated as the average of two determinations between about 5 and 30 min.

Ml. test solution present in 3 ml. substrate-enzyme mixture	Degradation rate VU	Observed titre of test solution VU/ml.
$2.50 \cdot 10^{-2}$	21.5	860
$1.25 \cdot 10^{-2}$	12.4	992
$6.25 \cdot 10^{-3}$	6.25	1 000
$3.13 \cdot 10^{-3}$	3.27	1 046
$1.56 \cdot 10^{-3}$	1.58	1 008
$7.83 \cdot 10^{-4}$	0.781	1 000
$3.92 \cdot 10^{-4}$	0.371	952
$1.96 \cdot 10^{-4}$	0.181	927
	Average ($n=8$)	973 ± 15 VU/ml.

The error of a single determination, σ_i, was calculated from 25 double determinations at different enzyme concentrations using the same substrate for the two tests of each pair, according to the formula $\sigma_i = \pm\sqrt{\frac{\sum d^2}{2n}}$, where d is the difference between two observations in per cent of their mean, and n is the number of double determinations. For the range of degradation rates 0.2–12 VU a value of ± 5.2 % was found.

The reproducibility of the assay with different hyaluronates was somewhat less accurate. For a degree of purity such as that of the preparations used in the present study, the error of the method was of the order of ± 10 %. Thus it is convenient to use the same preparation for routine determinations. A suitable preparation may be selected by testing different hyaluronates with a fixed amount of hyaluronidase and choosing a preparation giving high titre, indicating a high degree of purity. The preparation should also be tested by plotting $\frac{1}{[\eta]}$ against the reaction time (see fig. 12), since the accuracy of the assay will obviously be greater if this relationship is linear for a wide range of viscosities. Different preparations were found to vary to some extent in this respect, which can probably be ascribed to varying degrees of polydispersity.

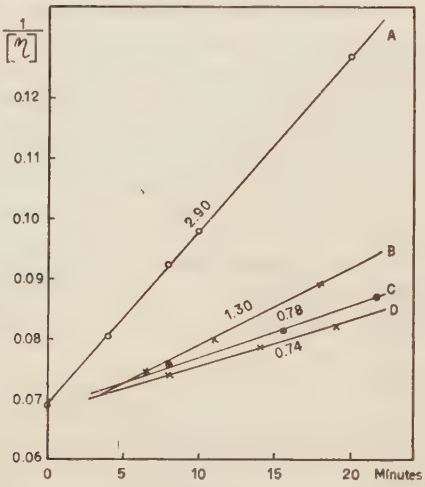

Fig. 16. Inhibition of hyaluronidase by blood serum.

A = hyaluronate only.
B = hyaluronate + serum in dilution 1:20.
C = hyaluronate + serum in dilution 1:10.
D = hyaluronate + serum in dilution 3:10.
The numerals in the fig. indicate degradation rates in VU. The viscosities were corrected for the fractions due to the serum added.

As is evident from fig. 16, the procedure may also be used for direct determination of hyaluronidase inhibitors such as that in blood serum.

Toluene has been shown to abolish the effect of the serum factor (Adner, 1948). It should thus be possible to measure this type of inhibition also in synovial fluid by determining the difference in degradation rates in the presence and absence of this agent.

In the presence of higher concentrations of serum, and in tests with synovial fluid as substrate, correction for the non-hyaluronate fraction of the viscosity should be made. For this the end-value of the enzymatic hydrolysis was determined after addition of 0.1 ml. of a hyaluronidase solution, containing about 500 units, to the test sample. Since an erroneous estimation of the end-value evidently influences the assay relatively more at low viscosities, the degradation rate should preferably in such cases be computed from determinations in the range of η_{sp} above 2–3.

DEGRADATION RATES WITH SYNOVIAL FLUIDS AS SUBSTRATE

From fig. 17 it is evident that the relationship between $\frac{1}{[\eta]}$ and the reaction time on degradation with hyaluronidase of synovial fluids of different types, is as a rule approximately linear, in analogy with the conditions shown for substrates of isolated hyaluronate.

This was also valid for the normal fluid of fig. 17, which contained highly polymerized polysaccharide ($[\eta] = 50.9$).

The essential constancy of the degradation rate, computed from eq. (13), in this and other fluids of high viscosity, was notable with regard to the rapid initial drop in relative viscosity observed in such fluids.

It appears probable that the degradation rate would have been different in the initial stage if opening of labile interparticle bonds (Meyer, 1950) or splitting of a polysaccharide-protein complex (Ogston and Stanier, 1950) were the chief cause of the initial rapid drop in viscosity.

The fact that the shape of the degradation curve was similar for both normal and pathological synovia as well as for pure hyaluronate gives

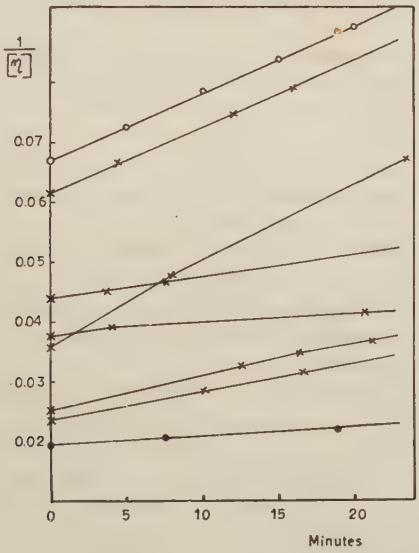

Fig. 17. Initial degradation rates at constant hyaluronidase concentration using hyaluronate and synovial fluids of different types as substrate.

○ = hyaluronate in phosphate-NaCl of pH 7.0.
● = normal synovial fluid.
× = pathological synovial fluid.

Table 16. Relation of degradation rate to intrinsic viscosity at constant hyaluronidase concentration.

The averages of 'relative activity' given refer to the rates obtained with solutions of hyaluronate at the same concentrations in standard buffer. The material was classified according to intrinsic viscosity, as shown in the table.

Intrinsic viscosity	No.	Relative activity %
>30	10	36.9
<30 >23	12	39.2
<23	12	59.4
Total	34	45.7

some support to the view that the synovial polysaccharide and isolated hyaluronic acid are homologous polymers although of a varying degree of polymerization.

On the other hand, the actual rates obtained with synovial fluids varied greatly, and were usually lower than the corresponding values for substrates of isolated hyaluronate. In order to investigate whether there were any differences in this respect between different types of joint fluids, the initial degradation rates (within about 20 min.) were determined in a number of undiluted fluids using constant enzyme concentration. The results are given in table 16, the observed rates being expressed as percentages of the values obtained with pure hyaluronate of the same concentrations as substrate. The latter values were obtained by interpolation from a diagram relating the apparent activity to the substrate concentration (see fig. 14, page 160).

As is seen from table 16, there was a tendency to higher rates the lower the intrinsic viscosity, although the variation was too great to allow of any conclusions. A comparison with the data of table 18 would imply that the inverse relationship between degradation rate and intrinsic viscosity was only apparent.

Further analysis of the relative degradation rates was of interest in some pathological fluids where, as will be shown on page 208, chapter IX, a marked decrease in intrinsic viscosity of the polysaccharide seemed to be associated with acute damage to the articular cartilage. In such cases of traumatic and degenerative arthritis, admixture of sulphate-containing polysaccharides might well have occurred on destruction of detached cartilage fragments. Regarding the much lower viscosity of chondroitin sulphate in comparison with that of hyaluronate of the same concentration (Blix and Snellman, 1945) it follows that presence of chondroitin sulphate might cause an apparent decrease in intrinsic viscosity of the synovial polysaccharide, as assayed in this investigation,

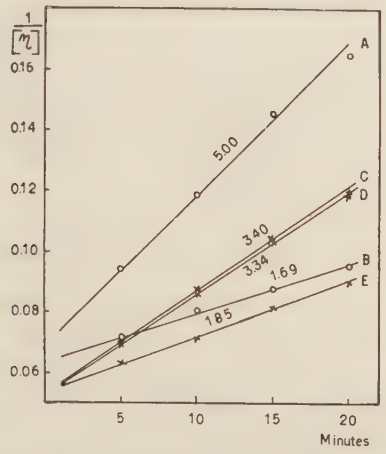

Fig. 18. Inhibition of hyaluronidase by chondroitin sulphate.

A = 0.1 % hyaluronic acid in standard buffer.
B = 0.1 % hyaluronic acid solution containing 0.225 mg. chondroitin sulphate (from articular cartilage) per ml.
C = joint fluid diluted with standard buffer to a hyaluronic acid concentration of 0.1 %; toluene added.
D = as C, no toluene added.
E = as D; 0.225 mg. chondroitin sulphate added per ml.
The numerals in the fig. indicate reaction rates in VU.

since both isolated (Meyer *et al.*, 1941; Madinaveitia and Stacey, 1944) and naturally-occurring (Meyer and Rapport, 1950) chondroitin sulphate from cartilage are also hydrolysed by testicular hyaluronidase.

It appears that if the above explanation of the decreased intrinsic viscosity were a factor of importance in some pathological fluids the inhibitory action of chondroitin sulphate upon the enzymatic degradation of hyaluronate might be detected by comparing the degradation rates at fixed enzyme concentration in different types of fluids.

The marked inhibitory action of even small amounts of chondroitin sulphate on the degradation of hyaluronate solutions or naturally-occurring fluids is illustrated by the data in fig. 18 and table 17.

On increasing the concentration of chondroitin sulphate the inhibition was only slightly more marked.

Since the marked inhibitory action brought about by adding chondroitin sulphate was retained in the experiments with joint fluid as substrate, significant amounts of sulphate-containing polysaccharides could hardly have been present in this fluid.

The slightly lower degradation rate as compared with hyaluronate solutions probably cannot be ascribed to the presence in the fluid of

Table 17. Inhibition of hyaluronidase activity in different substrates.

The table gives the results of two series of experiments (see also fig. 18) at different enzyme concentrations. The polysaccharide preparations (hyaluronate from umbilical cord and chondroitin sulphate from articular cartilage) were dissolved in phosphate-NaCl of pH 7.0. The joint fluid was diluted with salt-buffer to a polysaccharide concentration of 0.1 g. per 100 ml. The intrinsic viscosity of the sample was 19.8, compared with 15.1 for the hyaluronate used. All inhibition values refer to the rates with 0.1 % hyaluronate as substrate.

	Substrate	Apparent degradation rate VU	Inhibition %
Enzyme concentration I	A. Hyaluronate, 1 mg./ml.	5.00	—
	B. As in A, + chondroitin sulphate, 0.225 mg./ml.	1.69	66
	C. Diluted joint fluid	3.34	33
	D. As in C, + toluene	3.40	32
	E. As in C, + chondroitin sulphate, 0.225 mg./ml.	1.85	63
Enzyme concentration II	A. Hyaluronate, 1 mg./ml.	2.72	—
	B. As in A, + chondroitin sulphate, 0.114 mg./ml.	1.11	59
	C. As in A, + chondroitin sulphate	0.81	70
	D. Diluted joint fluid	2.05	25

Table 18. Variation in apparent activity of hyaluronidase in tests with different types of joint fluid as substrate.

For explanation of the groups see text. Groups I and II were classified according to intrinsic viscosity, the averages of intrinsic viscosity and relative activity being given for each sub-group. The latter are the actual rates observed, expressed in percentages of the rates obtained using a substrate of pure hyaluronate in standard buffer at the same concentrations of polysaccharide.

Group	No.	Intrinsic viscosity	Relative activity %
I. Traumatic arthritis, degenerative joint disease	9	38.2	38.8
	9	25.4	46.8
II. Rheumatoid and infectious arthritis	8	26.6	49.7
	8	17.6	47.9

Difference between means of groups I and II 6.0 ± 10.4 %.

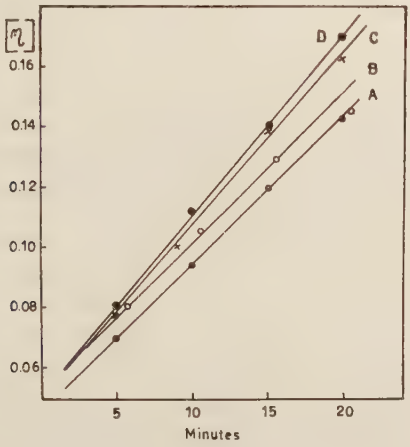

Fig. 19. Relative degradation rates at fixed hyaluronidase concentration for different substrates.

A = 0.05 % hyaluronate in phosphate-NaCl of pH 7.0.
B = undiluted synovial fluid containing 0.05 % hyaluronic acid.
C = synovial fluid diluted with salt-buffer to a hyaluronic acid concentration of 0.05 %.
D = same as C, toluene added.

inhibitors of the type found in blood serum, to judge from the fact that toluene addition caused no appreciable change in the degradation rate.[1]

One further experiment with two fluids of traumatic type which also exhibited marked decrease in intrinsic viscosity, is given in fig. 19.

The rates are seen to be nearly identical in the synovial fluids and the hyaluronate solution, which seems to exclude the presence of hyaluronidase-sensitive, sulphate-containing polysaccharides in these cases.

In table 18 the data of table 16 have been arranged in aetiological groups, following the classification used by Bauer and associates (see page 184). Group I thus includes fluids from traumatic and degenerative arthritis, and group II all types of infectious and rheumatoid arthritis.

The somewhat lower average rate in the sub-group of high intrinsic viscosity of group I can probably be ascribed to inhibitors due to blood admixture, since most of these effusions had been aspirated shortly after injuries to the joint. Although the variation was great in all groups, the approximately similar average rates indicated no gross differences between different types of pathological fluids with regard to the possible presence of cartilage polysaccharides.

[1] Of some fluids tested with regard to the effect of toluene on the degradation rate with hyaluronidase, two samples from traumatic effusions showed 10–22 % higher rates in the presence of toluene, which can probably be ascribed to blood admixture.

CHAPTER VI

Discussion. On the State of the Hyaluronic Acid in Synovial Fluids

The detailed chemical composition of the naturally-occurring hyaluronic acid has so far not been definitely established.

In this investigation a hexosamine content corresponding to 40 % for Na-hyaluronate was assumed, which is in close agreement with values reported by several workers for hyaluronate from synovial fluid (Meyer *et al.*, 1939; Ropes *et al.*, 1947; Ogston and Stanier, 1950). This value is however lower than calculated from the formula generally accepted for hyaluronic acid (i.e. 44.6 % for the sodium compound). Blix and Snellman (1945) suggested that the discrepancy might be ascribed to oxidative degradation occurring during preparation. According to Skanse and Sundblad (1943) this type of break-down is accompanied by a fall in glucosamine and an increase in acid groups, possibly due to formation of glucosaminic acid. The findings by Hadidian and Pirie (1948) of a lower glucosamine content, and by Meyer (1948) of a higher number of free carboxyl groups, in hyaluronate preparations of low mean polymerization would evidently both be consistent with such a view. So far, however, attempts by the present author to demonstrate the actual presence of hexosaminic acid in hyaluronate preparations have been unsuccessful. Furthermore, glucosamine values close to the theoretical ones have recently been reported for hyaluronate isolated from tumour fluid (Blix, 1951 A, B), and from synovial fluid (Ogston and Stanier, 1950), in spite of marked loss in viscosity during the isolation procedure.

Ogston and Stanier (1950) suggested, on the basis of glucosamine and nitrogen analyses of a protein-polysaccharide complex isolated from normal synovia, that some 10 % might consist of material free from both nitrogen and glucosamine. It seems however questionable whether sufficiently reliable absolute figures for hexosamine may be obtained to justify definite conclusions to be drawn solely from analysis data.

Structural differences from the normal may of course be present in the altered hyaluronic acid of pathological fluids, but so far no observations indicating this have been reported.

It is also possible that hyaluronidase-sensitive polysaccharides other than hyaluronic acid may be present in the fluids. Such compounds would then be included in the value obtained on assaying the 'hyaluronic acid' by the method described.

The ground-substance of connective tissue thus appears to consist of about equal proportions of sulphated and sulphate-free polysaccharidic acids (see Meyer, 1947; Pearce and Watson, 1949). Further, destruction of cartilage is a common feature of various types of arthritic disease.

In some pathological fluids a marked decrease in intrinsic viscosity was apparently associated with the event of acute damage to the articular cartilage (see chapter IX, page 208). In such cases of traumatic and degenerative arthritis liberation of chondroitin sulphate might well be thought to occur on destruction of detached cartilage fragments.

However, to the author's knowledge, the presence of significant amounts of chondroitin sulphate has not been demonstrated either in normal or pathological synovia.

Consistent with this were the results of a few sulphate analyses performed on synovial fluids. Thus no ester-bound sulphate could be demonstrated in a sample of normal fluid from horse, or in two samples from human knee effusions of traumatic origin. In a fluid from arthrosis deformans and two fluids from rheumatoid arthritis, between 0.3 and 0.5 mg. ester-bound sulphur per 100 ml. fluid was found, which implied that from 2–7 % of the total polysaccharide content of these fluids might consist of chondroitin sulphate. However, even after prolonged treatment with hyaluronidase, this sulphate could not be eliminated by dialysis, and the corresponding polysaccharide, if present, would thus not be included in the estimation of hyaluronic acid.

Nor did results of studies on hyaluronidase inhibition in synovial fluids (see chapter V, page 165) indicate the presence of significant amounts of chondroitin sulphate even in such pathological fluids where this might well have been suspected.

Summarizing the above discussion it may be stated that the method described in chapter III appears to provide reliable values for the hyaluronic acid concentration in naturally-occurring fluids.

The question whether the variations of the intrinsic viscosity found by the method described in chapter IV may be used as a quantitative measure of the hyaluronic acid changes in the fluids deserves further consideration.

It has not been settled so far whether the much lower viscosity of isolated, compared with naturally-occurring, hyaluronic acid is entirely to be ascribed to degradation during the isolation procedures, or whether the difference should be otherwise explained.

The results given in chapter IV seem to favour the former idea. Thus the viscosity/concentration relationship was approximately the same for solutions of pure hyaluronate and joint fluids containing polysaccharide

of widely varying intrinsic viscosities. The viscous behaviour in this respect was on the other hand found to be different for chondroitin sulphate and alkali-degraded hyaluronate in spite of the closely related chemical composition.

The studies on anomalous viscosity showed on the other hand some differences. Thus marked shear-dependence was only present in the naturally-occurring fluids, and could not be demonstrated in solutions of hyaluronate of low mean polymerization. This discrepancy, however, might well be explained by differences in average molecular weight (see figs. 9 and 10, pages 150 and 151).

Ogston and Stanier (1951) consider the polysaccharide molecule of normal synovial fluid to have a highly flexible long-chain structure. A comparison with the viscous behaviour reported for a synthetic polyelectrolyte of probably similar molecular dimensions in a recent paper by Alexander and Hitch (1952) is therefore of interest. These authors studied polymethacrylic acid of varying degree of ionization in a range of molecular weights of the order 10^5–10^6.

The viscous anomalies observed for this polymer appear to be of the same type as those shown by hyaluronic acid. Thus polymethacrylate of high (i.e. $2 \cdot 10^6$), but not of low (i.e. $2 \cdot 10^5$), molecular weight showed a very pronounced anomalous viscosity. The shear-dependence was of a magnitude which could not be explained by hydrodynamic orientation.

The authors concluded that the flow anomalies result from reversible formation of a network of chains which is progressively broken down by an increasing gradient.

Consequently factors which influence molecular interaction will also influence anomalous viscosity. Such factors include both size and shape of the molecule. For a polyelectrolyte with long-chain structure, the latter will depend on the rigidity of the chain as well as on the degree of electrostatic repulsion between ionized (carboxyl) groups. Thus the viscous anomaly is reduced, owing to a coiling up of the molecule, by reducing the degree of ionization of the polymer, or by increasing the ionic strength.

By analogy, the similar viscous behaviour of synovial fluid probably chiefly depends on molecular interaction. The much higher degree of anomalous viscosity observed in synovial fluids at fixed pH and ionic strength compared with solutions of pure hyaluronate (see chapter IV, figs. 5–10) might then well be explained by greater average molecular weight of the naturally-occurring polysaccharide.

Ogston and Stanier (1951) concluded that the polysaccharide molecule occurs in normal synovial fluid as a nearly completely coiled chain. This view is based on the finding that the viscosity/concentration ratio was nearly independent of the velocity gradient at high degrees of dilution. The high viscosity was accounted for by the assumption that the particles are very highly solvated. Their results of studies on birefringence

of flow and on the variation of the sedimentation constant with concentration were consistent with the theory of a low axial ratio.

The present studies indicated, however, that for hyaluronic acid of high mean polymerization the flow anomalies would not be of negligible magnitude even at infinite dilution. Thus the observed shear-dependence of the intrinsic viscosity (see chapter IV, figs. 8–10) was considerably greater than reported by Ogston and Stanier.

The possibility should further be considered, whether the molecular interaction is of purely mechanical type, favoured by great particle-hydration, or whether the polymer chains are also held together at points of intersection by some type of labile intermolecular linkages. Jeanloz and Forchielli (1950) have concluded that the chains form loose aggregates of high molecular weight in naturally-occurring fluids. That the carboxylic groups may be involved in the interconnexions between particles was suggested by the observation of a fall in viscosity under the action of diazomethane. A sudden fall in viscosity observed at high temperatures was also interpreted as due to the breaking of interparticle linkages.

Meyer (1948) has suggested that the high viscosity of naturally-occurring fluids is in part due to anhydride linkages between the chains. This hypothesis was rejected by Jeanloz and Forchielli (1950), who succeeded in preparing hyaluronate of very high viscosity with a sodium content close to the theoretical value.

A role of the proteins of naturally-occurring fluids in interconnexions between the particles would be one further possibility. Electrophoretic studies on synovial fluid and synovial mucin (Blix, 1940; Hesselvik, 1940; Ropes *et al.*, 1947) have, however, shown that the polysaccharide migrates completely separated from protein, suggesting that the hyaluronic acid occurs free in the fluid. Blix (1940) further found the nitrogen/glucosamine ratios to be nearly the same for the separated fast-moving component and the polysaccharide obtained by chemical isolation.

In spite of the electrophoretic evidence[1], a loose complex-formation with proteins has been assumed for naturally-occurring hyaluronic acid by Ropes *et al.*, 1947. This was based on differences in precipitability, behaviour on ultrafiltration, and effect of pH on the viscosity, observed between synovial mucin and pure hyaluronate. A similar view was held by Ogston and Stanier (1950, 1951). These authors found in fact that the removal of the protein component was invariably associated with a disappearance of the characteristic anomalous viscosity shown by the naturally-occurring complex.

It seems, however, that the change in viscosity must not necessarily be ascribed to a dissociation of such a complex. The responsible factor

[1] Ogston and Stanier (1952) have recently, however, reported unsuccessful attempts to separate protein-free hyaluronic acid from synovial fluid by means of electrophoresis.

may equally have been a degradation of the polysaccharide component by the preparation procedures. The observation by Ropes *et al.* (1947) that complete tryptic digestion does not appreciably affect the viscosity, as well as that by Gunter, 1949, that trypsin causes only insignificant changes in the 'Spinnbarkeit' of normal synovia, seems in fact to exclude a role of the proteins in the viscous behaviour of synovial fluid.

According to Meyer (1950), pure hyaluronate of very high viscosity may even give typical mucin-clots in the presence of proteins, indicating that the different behaviour on precipitation with acetic acid ordinarily found for hyaluronate-protein mixtures compared with normal fluids is probably also entirely due to the difference in molecular weight.

Summarizing the above discussion it may be stated that most differences in physico-chemical behaviour observed between isolated compared with naturally-occurring hyaluronic acid may readily be referred to a degradation of the polysaccharide during the isolation procedures.

The results given in chapter IV and V show that the viscous behaviour, as well as the shape of the viscosity reduction curves after hyaluronidase addition, are closely similar in synovial fluids of all types and in solutions of pure hyaluronate. It therefore seems justifiable to conclude that the naturally-occurring polysaccharide and isolated hyaluronic acid are members of the same polymer-homologous series.

Under standardized measuring conditions the intrinsic viscosity may then be used as a relative measure of the average degree of polymerization.

Whether it is possible to utilize the numerical values of $[\eta]$ provided for the determination of particle dimensions has so far not been established.

Difficulties in this respect arise from the marked anomalous viscosity of highly polymerized hyaluronic acid. Owing to this, accurate values for the viscosity at either zero or infinite velocity gradient cannot conveniently be obtained with the aid of capillary viscometers (see chapter IV, figs. 5 and 6).

A comparison of intrinsic viscosities, in spite of marked variation in degree of anomalous viscosity, could on the other hand be made with satisfactory accuracy by the method described in chapter IV, using viscometers of fixed dimensions.

A comparison of the results obtained for synovial fluids with the data given by Blix and Snellman (1945) for hyaluronate preparations seemed of interest. Assuming a direct proportionality between particle length and intrinsic viscosity at zero gradient, the observed range of $[\eta]$ for normal synovial fluids (see table 19, page 177) would, after approximate correction to zero gradient (see fig. 10, page 151), imply molecular weights of the order of $2\text{–}6 \cdot 10^6$ for the naturally-occurring polysaccharide.

Although it is questionable whether this comparison is justified, it may be noted that values of about the same magnitude have been ar-

rived at by other workers from essentially different theoretical considerations. Thus Ogston and Stanier (1951) give (unsolvated) molecular weights of about $8–9 \cdot 10^6$ for a polysaccharide-protein complex from normal synovial fluid. Similarly, Jeanloz and Forchielli (1950) assume weights of several million for the particles of naturally-occurring fluids.

Finally, by analogy, values of this order of magnitude may be considered probable from a comparison with reported data for other chain-polymers of polyelectrolyte character showing the characteristic structural viscosity of normal synovial fluid.

CHAPTER VII

Chemical Findings in Normal Synovial Fluids

The results of analysis of 24 fluids from different animal joints, and two samples from normal human knee joints, are compiled in table 19.

It is apparent from the table that both hyaluronic acid concentration and relative viscosity varied widely, approximately in inverse proportion to the fluid volume. The variation with regard to the protein content was less marked, and no obvious relation to the fluid volume could be found. The average intrinsic viscosity is seen to be of about the same magnitude in the various groups of fluids. The variation in intrinsic viscosity of the fluids from calves was relatively small ($\sigma = \pm 5.3$) compared with the fluids from horses ($\sigma = \pm 12.9$). This discrepancy is probably associated with the varying age of the animals in the latter group. The presence of osteo-arthritic joint changes could thus be expected in some cases, and, as will be shown below, such changes are usually accompanied by lowered intrinsic viscosity of the synovial polysaccharide.

The analytical findings in the fluids of animal origin are in keeping with those reported by other authors, as far as viscosity and protein concentration are concerned. This applies in part also to the hyaluronic acid concentration as roughly estimated from reported values of mucin concentration or mucin glucosamine. (Ropes *et al.*, 1939, 1940; Bauer *et al.*, 1940; Davies, 1944. Further references may be found in the paper by Ropes *et al.*, 1947. The early literature is fully covered in the monograph by Kling, 1938.)

The results of analysis for the human fluids were in close agreement with the rest of the material of table 19, if consideration is taken for the fluid volume. The fluid findings were essentially the same in part of the pathological material to be discussed in the following chapter, viz. in cases of slight joint disability after minor injuries (group 1 b, page 181) with insignificant or no increase in fluid. Thus average protein and hyaluronic acid concentrations of 2.5 and 0.264 g. per 100 ml. respectively, and an average intrinsic viscosity of 39.0, were found for 7 fluids of a total volume of ≤ 2 ml. One further sample aspirated from the unaffected knee joint of a case of rheumatoid spondylitis without involvement of peripheral joints (table 25, page 195) may perhaps also be regarded as 'normal fluid'.

Table 19a. Chemical findings in normal synovial fluids of human and animal origin.

In several instances the analyses were made on conjoined samples from different joints of the same animal. The number of joints is thus not identical with the number of fluids given in the table. The averages of protein concentration, hyaluronic acid concentration, and intrinsic viscosity, are given. Total protein was not determined in all samples; the figures in parentheses give the number of protein determinations. Approximate values of fluid volume are included for comparison. Relative viscosities were not directly determined in fluids of high viscosity, but could be calculated from the values of hyaluronic acid content and intrinsic viscosity, as explained in the text.

Species	Number of fluids	Fluid volume, ml.	Hyaluronic acid, g. per 100 ml.	Relative viscosity	Intrinsic viscosity	Total protein, g. per 100 ml
Human[1] . .	2	½—1	0.297	>300	39.3	2.8 (2)
Calf	11	1—3	0.193	15—>300	45.0	1.7 (7)
Ox	1	20	0.045	4	40.4	1.2
Horse	10	5—40	0.056	3—29	51.5	1.9 (5)
Rabbit[2] . .	2	<0.1	0.389	>300	37.4	3.6 (2)

[1] These fluids were taken from the unaffected knee joints of two patients with unilateral traumatic arthritis.

[2] These fluids were mixed samples each from 5 knee joints, collected by means of a pipette. The author is indebted to Dr. Ragnar Ekholm, Institute of Anatomy, Medical School, Gothenburg, for sending these fluids for analysis.

Table 19 b. Average values for normal fluids.

Total no. of normal fluids, 26	Hyaluronic acid, g. per 100 ml.		Intrinsic viscosity		Total protein, g. per 100 ml.	
	$M \pm \varepsilon (M)$	σ	$M \pm \varepsilon (M)$	σ	$M \pm \varepsilon (M)$	σ
	0.157 ± 0.022	0.114	46.3 ± 1.9	9.7	2.1 ± 0.2 (17)	1.0

The hyaluronic acid concentrations, as determined by a turbidimetric method, reported for normal fluids from the human knee joint by Ragan and Meyer (1949) were on an average lower than those given above. This is no doubt associated with the fact that the fluids were obtained post mortem, the amount often being increased owing to peripheral oedema. Calculation of intrinsic viscosity from the hyaluronic

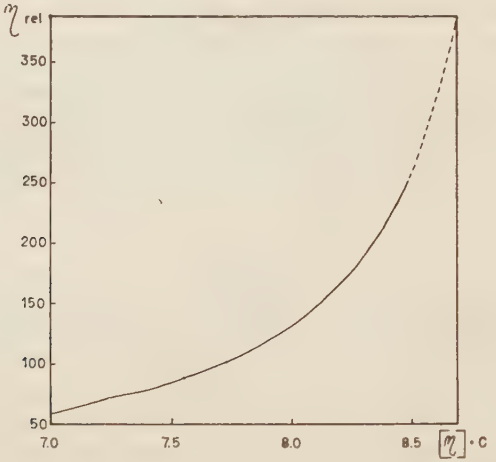

Fig. 20. Relative viscosity as a function of intrinsic viscosity, $[\eta]$, and concentration of hyaluronic acid, *c*.

The diagram gives the relationship of eq. (5), page 140, valid both for hyaluronate solutions and naturally-occurring fluids. Uninterrupted line = experimentally tested range of η_{rel}.

acid concentration and relative viscosity given by these authors produced a range of values essentially similar to the normal fluids in table 19. The conditions under which the viscosity determinations were made were, however, too different from those in the present study to allow of any but a rough comparison (see also Ragan, 1946).

The wide variation in hyaluronic acid concentration observed in all types of normal fluids should probably rather be ascribed to varying degree of dilution of the hyaluronic acid produced than to varying 'secretion capacity' of the synovial tissue. Some indirect support to this view was the finding that also in the normal or probably normal human fluids the hyaluronic acid concentration varied roughly in inverse proportion to the amount of fluid present in the joint cavity (see fig. 30, page 210). The degree of dilution probably also changes continuously in the same joint depending on its functional state, since the amount of fluid has been found to increase during activity (Ekholm and Norbäck, 1951). Hyaluronic acid concentrations between 0.2—0.4 g. per 100 ml. may, however, be accepted as the probable range for normal synovial fluid from the human knee joint, which corresponds to the findings in fluids of a total volume of up to about 2 ml., representing the limit of normal variation given by Coggeshall *et al.* (1940).

Regarding the comparatively slight variation in intrinsic viscosity (see table 19) it is evident that the variation in fluid viscosity is mainly due to the varying polysaccharide concentration. Direct determination of

the viscosity in the range of concentrations found in normal human fluids is subject to great errors arising from the marked shear-dependence of the viscosity at high polysaccharide concentrations (see table 11, page 147). Equation (5), page 140, may however also be used to calculate the relative viscosity of the undiluted fluid from viscosity determinations made on dilutions. This is advantageous, since comparisons can be made under standardized measuring conditions. The relationship between η_{rel} and the product of intrinsic viscosity and concentration is illustrated graphically in fig. 20 for the experimentally tested range of viscosities.

From fig. 20 a minimum value of several hundred is obtained for the range of concentrations and intrinsic viscosities found in the human fluids.

The observed range of $[\eta]$ would imply molecular weights of the order of several million for the polysaccharide of normal fluid (see discussion in chapter VI).

CHAPTER VIII

Chemical Findings in Pathological Synovial Fluids

The material available for this investigation was rather heterogeneous, the samples having been referred for analysis from several different clinics, most of them special departments for rheumatology and orthopaedics. Nearly half the cases were obtained from out-patient departments, and the patients had often not been subjected to all the routine tests which might have been desirable for full classification of the cases.

The information which could be obtained when the joint was aspirated was collected by means of a specially-designed questionnaire recording some essential details of the history, in addition to the results of clinical examination and selected laboratory data. The preliminary diagnoses were later checked in the hospital records, except in a few cases where this for different reasons was not possible. These cases were however included, using the preliminary data as a basis for the clinical classification.

The results of this study are based upon analyses of fluid[1] aspirated from knee joints of 206[2] patients, in addition to a few samples from bursae.

In the statistical treatment of the series, presented in this chapter, only the results of analysis of the first sample were included in the case of patients who had been repeatedly tested at intervals. If both knee joints had been simultaneously aspirated the samples were analysed separately and the average values used. The remaining results were partly used for computing the individual variation of the analytical findings, and were in part also included in the study of the influence of different factors upon the tests (see chapter IX).

[1] In some instances the amount of fluid which could be aspirated from the joint was only insignificantly above normal, or even within the limits of normal variation given by Coggeshall *et al.* (1940). Although not 'effusions' in the proper sense of the word, these cases were included in the statistical treatment of the series, the relation of the tests to the degree of effusion being discussed separately in the next chapter.

[2] 15 cases had to be excluded either because of unsatisfactory results of analysis, in most cases due to bacterial contamination of the samples, or because insufficient clinical data were available. Three cases showing intrinsic viscosities of about 100, probably due to inaccurate glucosamine analyses, were excluded.

ARRANGEMENT OF THE MATERIAL

A strict classification of the cases, following the diagnosis, had to be abandoned in several instances because of the smallness of the series. However, 177 of the cases were conveniently classified as shown below, the remaining 29 cases being discussed separately after the survey of the main series (see page 194).

The cases were arranged as follows.[1]

Group 1. Traumatic arthritis. This group was divided into two subgroups after the following principles.

Group 1a contained 20 cases of traumatic arthritis following direct injury to the joint. No history of previous joint disability was given, and the available clinical data revealed no indication of incidental joint disease. Most specimens were aspirated within a few days of a sprain or contusion. Two cases were diagnosed as 'post-traumatic synovitis'. In those cases where recurrent effusion necessitated repeated aspiration of the joint before healing occurred, the analysed sample was that obtained on the first occasion (with the exception of 3 cases, where fluid strongly admixed with blood had been removed immediately after the injury).

Group 1b. In most of the 20 cases in this group the arthritis was not due to acute, severe trauma, but was secondary to repeated, often minor, injuries to the joint, sometimes over a considerable period. The common feature of the clinical picture was, however, that, on the basis of history and local signs, the presence of lesions of the semilunar cartilages had been suspected. The samples analysed were taken in connexion with arthrography, which was performed in 18 of the cases. The arthrograms failed to confirm the diagnosis in all cases, although findings which might have been indicative of slight damage to the semilunar cartilages were reported in a few cases. The degree of effusion was usually insignificant except where aspiration was done within some weeks of an acute sprain. Recurrent dislocation of the patella was reported as the cause of the joint disturbance in one case.

From group 1 were excluded some forms of traumatic arthritis and allied conditions, according to the above definition. These cases are briefly discussed on pages 196—198, except for those included in groups 2 and 3 as defined below. Only cases with negative radiograms were included in group 1. Several cases of uncomplicated sprains had, however, not been examined radiologically (see page 219).

Group 2. Traumatic arthritis associated with lesions of the semilunar cartilages. As in group 1b the 15 samples in this group were collected in connexion with arthrography, which revealed lesions of the internal semilunar cartilage in 13, and of the external cartilage in 2, cases.

[1] The nomenclature proposed by the New York Rheumatism Association (Hench *et al.*, 1948) has as much as possible been followed.

The diagnoses were confirmed in the 10 cases operated on within a few months of arthrography. Radiological evidence of degenerative joint disease was reported in 4 of the cases. The period between injury and aspiration varied greatly, and the degree of effusion was insignificant in most cases.

Group 3. Degenerative joint disease. Under this heading were included 31 cases with a primary diagnosis of 'arthrosis deformans', 5 cases diagnosed as 'chondromalacia patellae', and 6 cases of traumatic arthritis where radiological examination had revealed degenerative changes of varying degree. These last-named cases were included in sub-group 3a, which also included all cases where an injury to the joint, although usually insignificant, marked the onset of the joint disability, or had apparently aggravated the disease. In 23 of the 42 cases (sub-group 3b) the joint disturbance and the appearance of effusion bore no obvious relation to trauma.

Many of the patients appeared to suffer from a generalized form of degenerative joint disease. The degree of effusion was only moderate in most instances. The distribution of the cases according to the stage of the disease, as revealed by the radiograms, can be seen from table 38, page 218.

Group 4. Rheumatoid arthritis. This comprised 40 cases with the primary diagnosis 'polyarthritis chronica'.[1] Most presented the clinical picture, regarded as typical of rheumatoid arthritis, of a symmetrical, progressive polyarthritis mainly involving small joints. This was also the case in a few patients in whom the diagnosis at the onset of the disease had been 'rheumatic fever'. Two cases of subacute polyarthritis following rheumatic fever were excluded, as were also a few where the diagnosis of rheumatoid arthritis had been questioned. These cases are discussed below (table 25, page 195), together with some of ankylosing spondylitis, psoriatic arthritis, and Reiter's syndrome (group 6). Some additional information about the distribution of the cases in group 4 according to the stage and activity of the disease will be given in the next chapter, which deals with the relation of the chemical tests to various clinical features.

Group 5. Arthritis of infectious or unknown aetiology. In the majority of the 40 cases collected under this heading the nature of the joint disturbance was not established, diagnoses of acute, subacute, or chronic, synovitis of unknown aetiology having been made. The arthritis had, however, been considered as due to specific infections in 6 of the cases (3 tuberculous, 2 gonococcal, and 1 syphilitic). Also with respect to available clinical data the group was very heterogeneous,

[1] A distinction between 'polyarthritis chronica primaria' and 'polyarthritis chronica secundaria' was made in some clinics. Of the cases of this group, only one had the latter diagnosis at the time of aspiration, but the condition was later diagnosed as 'polyarthritis chronica primaria'.

Table 20. Distribution of the cases among the groups according to sex and age.

Group	Number of cases			Age in years	
	Total	Men	Women	Range	Average
1. Traumatic arthritis	40	29	11	5—64	31
2. Lesions of the menisci	15	13	2	11—44	35
3. Degenerative joint disease	42	22	20	27—82	53
4. Rheumatoid arthritis	40	21	19	8—68	42
5. Arthritis of infectious or unknown aetiology	40	24	16	17—64	37

but was included in the survey of the main series for comparison with the other more homogeneous groups.

The group was subdivided as follows.

Group 5a. Miscellaneous forms of arthritis with systemic reactions. The common feature in the 20 cases was the presence of various constitutional reactions such as fever (present in 8 cases), and increased erythrocyte sedimentation rate ranging from 25—122 mm. per hour, average 49 mm. Most cases had acute arthritis of one or two large joints. The radiological findings varied from negative radiograms to bony destruction in the cases of tuberculous arthritis. Large effusions were present in most instances. The group has been loosely termed *'infectious arthritis'*.

Group 5b. In this group, *'unclassified arthritis'*, were collected the 20 cases over from group 5a. Systemic reactions were essentially absent in all these cases, to judge from the available clinical data. The joint disturbance was localized to the affected knee joint, except for two cases of chronic arthritis of both knee joints. Some cases presented only an insignificant degree of effusion. Negative radiograms were reported in the cases examined.

The arrangement of the series may in some respects seem artificial, especially with regard to the admittedly scanty clinical data in several cases, but it was considered sufficiently reliable for the purpose of illustrating the possible value of the proposed chemical tests in the differential diagnosis of joint disease. The analysis of the relationship of the degree of changes in the fluids to certain clinical features (see chapter IX) did not indicate that the principal results given below would have been influenced to any extent by more careful selection of the cases from a diagnostic point of view.

For the purpose of analysis of some of the results it was found better to arrange the series in two main groups, as proposed by Bauer and associates (see for example Ropes, 1952). Group I, of the 'Bauer' classification roughly corresponds to groups 1—3 ('traumatic arthritis' and 'degenerative joint disease'), and group II to groups 4 and 5a ('rheumatoid arthritis' and 'infectious arthritis').

The age- and sex-distribution of the cases is given in table 20.

FLUID FINDINGS IN GROUPS 1—5

TOTAL PROTEIN CONCENTRATION

The results of 120 determinations of total protein are given in table 21.

It can be seen from table 21 that the 'Bauer' classification (see above) is readily applicable also to this series as far as protein concentrations are concerned. Thus the average concentration of total protein was about the same in groups 1—3 (traumatic and degenerative arthritis, group I) and significantly higher in groups 4 and 5a (group II), but it is evident from fig. 21 that there is great variation within both groups.

Table 21 a. Concentration of total protein in pathological synovial fluids.

For definition of groups, see page 181.

Group	Number of cases	Total protein, g. per 100 ml.	
		$M \pm \varepsilon(M)$	σ
1 a. } Traumatic arthritis	17	4.3 ± 0.3	1.3
1 b. } Traumatic arthritis	13	3.7 ± 0.4	1.3
1 a + 1 b. } Traumatic arthritis	30	4.0 ± 0.2	1.3
2. Lesions of the menisci	11	4.0 ± 0.5	1.6
3 a. } Degenerative joint disease	10	4.5 ± 0.4	1.3
3 b. } Degenerative joint disease	12	4.2 ± 0.3	1.1
3 a + 3 b. } Degenerative joint disease	22	4.3 ± 0.2	1.1
4. Rheumatoid arthritis	33	5.1 ± 0.2	1.0
5 a. Miscellaneous forms of arthritis with systemic reactions	11	5.5 ± 0.3	1.0
5 b. Unclassified arthritis	13	4.2 ± 0.4	1.6
Group I fluids (groups 1—3)	63	4.1 ± 0.2	1.3
Group II fluids (groups 4 and 5 a)	44	5.2 ± 0.2	1.0

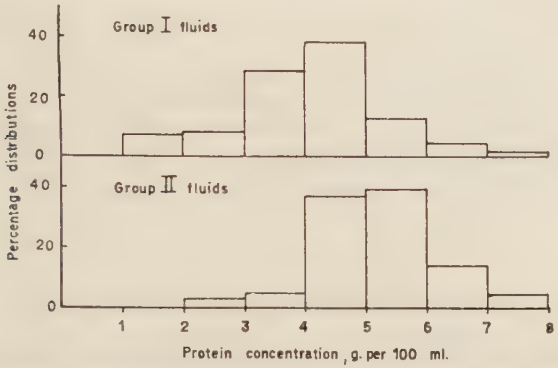

Fig. 21. Distributions of 63 group I fluids and 44 group II fluids according to concentration of total protein.

For definition of groups 1–5, see page 181.
Group I = groups 1–3, traumatic arthritis and degenerative joint disease.
Group II = groups 4 and 5a, rheumatoid and 'infectious' arthritis.

The value of protein determinations in differential diagnosis thus appears to be rather limited, fig. 21 showing that in the majority of fluids of both groups I and II values within the range 4—6 g. per 100 ml. were found. Levels below 4 g. per 100 ml. were, however, only found in about 6 % of the cases of rheumatoid or 'infectious' arthritis. Levels higher than 6 g. per 100 ml. were rarely found in traumatic and degenerative arthritis (about 6 % of the cases).

If the degree of effusion is also taken into consideration the test may be slightly more informative in some cases. It was found (see fig. 30, page 210) that the protein content was roughly proportional to the fluid volume in traumatic arthritis (group 1), whereas in degenerative or rheumatoid arthritis there was no apparent relationship between these values (table 34, page 211). Thus with fluid volumes of up to 10 ml. the highest value found in 10 traumatic fluids was 3.8 g. per 100 ml., in contrast with values below this level in only 4 out of 19 cases in groups 2—5. The finding of much protein in the fluid in traumatic arthritis with only an insignificant degree of effusion may

Table 21 b. Differences between groups.

Difference between	*D*	*t*	*P*
Groups 1 and 4.....	1.1	3.90	<0.001
3 and 4	0.8	2.83	<0.01
I and II.....	1.1	5.00	<0.001

thus be taken as some indication that the injury is not the sole cause of the joint disability.

In the case of large effusions the test is of less aid in differentiating between the groups, since in uncomplicated traumatic arthritis too the protein content may be greatly above normal. In degenerative joint disease almost normal protein levels were found in several cases in spite of large effusions, contrary to the findings in the rest of the series.

HYALURONIC ACID CONCENTRATION. RELATIVE VISCOSITY

The results of the determinations of hyaluronic acid and relative viscosity are given in table 22. In order to give an idea of the total amount of hyaluronic acid present in the joint cavity, the total volume, i. e. the amount of fluid which could be withdrawn from the joint, is

Table 22 a. Fluid volume, hyaluronic acid concentration, and relative viscosity, in pathological synovial fluids.

For definition of groups, see page 181. Maximum, minimum, and median, values of fluid volume and relative viscosity are given instead of the arithmetic means, because of the asymmetrical distribution. Relative viscosities were not directly determined in fluids of high viscosity, but could be calculated from the hyaluronic acid content and the intrinsic viscosity according to eq. (5). Levels above the experimentally tested validity range of eq. (5) are given as >300.

Group	Number of cases	Fluid volume, ml.		Hyaluronic acid, g. per 100 ml.		Relative viscosity	
		Range	Median	$M \pm \varepsilon (M)$	σ	Range	Median
1 a. Traumatic arthritis	20	5—100	40	0.102 ± 0.008	0.038	3—59	11
1 b. Traumatic arthritis	20	0.2—80	5	0.176 ± 0.017	0.075	3—>300	44
2. Lesions of the menisci	15	1—45	10	0.179 ± 0.016	0.063	3—>300	22
3 a. Degenerative joint disease	19	2—120	36	0.142 ± 0.012	0.054	3—66	13
3 b. Degenerative joint disease	23	1—95	40	0.139 ± 0.016	0.076	4—94	12
4. Rheumatoid arthritis	40	6—400	47	0.152 ± 0.010	0.061	2—34	11
5 a. Miscellaneous forms of arthritis with systemic reactions	20	8—170	60	0.134 ± 0.015	0.066	3—30	6
5 b. Unclassified arthritis	20	8—170	40	0.137 ± 0.013	0.059	4—89	14

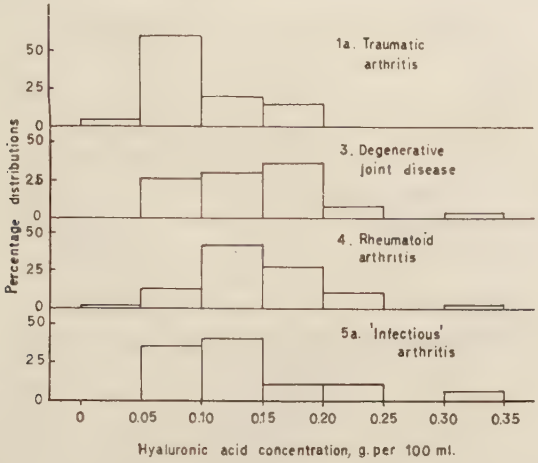

Fig. 22. Distributions of the fluids of groups 1a, 3, 4, and 5a, according to hyaluronic acid concentration.

For definition of the groups, see page 181. The distribution of the cases according to fluid volume was approximately the same in the four groups.

also recorded. As in the normal fluids (see chapter VII), the fluid volume and hyaluronic acid concentration in the traumatic fluids were inversely proportional (see fig. 30, page 210). Thus the average hyaluronic acid concentration was significantly higher in the traumatic groups (1b and 2) where the average fluid volume was small, than in group 1a. If, however, the groups with approximately similar fluid volume are compared (table 22b) a significantly lower average concentration was found in the traumatic fluids than in the fluids from rheumatoid and degenerative arthritis. The variation was, however, great within all groups (see fig. 22).

It appears that in the majority of the traumatic fluids levels below 0.1 g. per 100 ml. are found, whereas 65—85 % of the fluids in groups 3—5 show higher values. The actual concentration of hyaluronic acid

Table 22 b. Differences between groups with regard to average concentration of hyaluronic acid.

Differences between	*D*	*t*	*P*
Groups 1 a and 3	0.038	2.92	<0.01
1 a and 4	0.050	3.84	<0.001
1 a and 5	0.033	2.58	<0.05

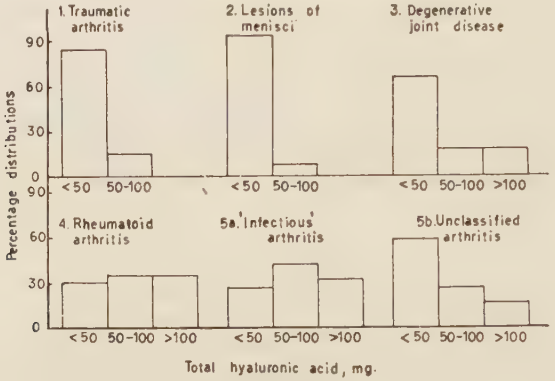

Fig. 23. Distributions of the cases of groups 1–5 according to total hyaluronic acid content of effusion.

is, on account of the great variation, less helpful in differentiating between the groups of fluids than is the total amount of hyaluronic acid contained in the effusion (see fig. 23).

Under normal conditions the total amount of hyaluronic acid present in the joint cavity does not exceed a few mg., except in animal fluids of large volume (see table 19, page 177). It is thus evident that there is often great increase in hyaluronic acid production by the synovial tissue under pathological conditions. The changes are, however, less marked in traumatic disorders than in rheumatoid and 'infectious' arthritis (fig. 23), where the hyaluronic acid content of large effusions may exceed 300 mg. The fact that values below 50 mg. were found in 83—93 % of the traumatic fluids of groups 1—2 compared with 65 % in the degenerative, and only 26—29 % in the 'infectious' and rheumatoid groups, is some indication that the test may be of value in differentiating between the group I and the group II types of effusion.

In the absence of qualitative changes in the hyaluronic acid the relative viscosity might provide a rough measure of the concentration of the polysaccharide. The data in table 22 show, however, that the average values of relative viscosity vary widely between groups with approximately the same content of hyaluronic acid. It is further evident that the relative viscosity is too variable to be of much value in differentiating between the groups, although low viscosities are commonest in fluids of infectious type. The finding of a high relative viscosity in a fluid is however some indication that the fluid is of group I type, as can be seen from the diagram in fig. 24.

Thus viscosities above 20 were found only in about 5 % of the fluids of rheumatoid and 'infectious' arthritis, whereas nearly half the cases in the traumatic and degenerative groups showed higher levels.

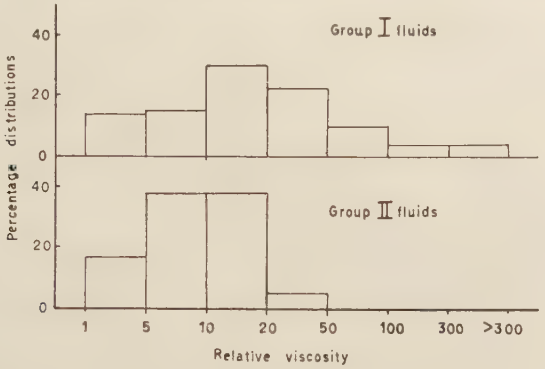

Fig. 24. Distributions of 97 group I fluids and 60 group II fluids according to relative viscosity.

Group I = groups 1–3, traumatic arthritis and degenerative joint disease.
Group II = groups 4 and 5a, rheumatoid and 'infectious' arthritis.
The class limits were chosen arbitrarily as shown in the fig., with the last class open (viscosities > 300).

INTRINSIC VISCOSITY

The best method of differentiating between the effusions of different groups is by calculating the intrinsic viscosity from the relative viscosity and the hyaluronic acid concentration, as described in chapter IV. The results are given graphically in fig. 25; table 23 shows the average values in the different groups.

Table 23 a. Intrinsic viscosity of the hyaluronic acid in joint effusions.
For definition of groups, see page 181.

Group	Number of cases	Intrinsic viscosity		Difference of M between sub-groups $D \pm \varepsilon(D)$
		$M \pm \varepsilon(M)$	σ	
1 a. Traumatic arthritis	20	39.1 ± 1.1	5.0	2.8 ± 2.0
1 b. Traumatic arthritis	20	36.3 ± 1.7	7.4	
1 a + 1 b. Traumatic arthritis	40	37.7 ± 1.0	6.4	
2. Lesions of the menisci	15	30.1 ± 1.8	6.8	
3 a. Degenerative joint disease	19	30.1 ± 2.2	9.4	0.5 ± 2.8
3 b. Degenerative joint disease	23	29.6 ± 1.8	8.9	
3 a + 3 b. Degenerative joint disease	42	29.8 ± 1.4	9.0	
4. Rheumatoid arthritis	40	26.3 ± 1.2	7.8	
5 a. Miscellaneous forms of arthritis with systemic reactions	20	23.9 ± 1.8	7.9	9.3 ± 2.7 $P < 0.01$
5 b. Unclassified arthritis	20	33.2 ± 2.1	9.3	

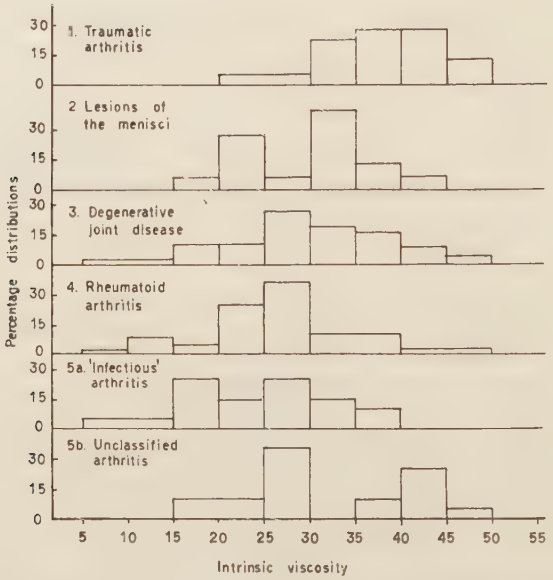

Fig. 25. Distributions of the fluids of groups 1–5 according to intrinsic viscosity.

Table 23 b. Differences between groups.

Difference between	*D*	*t*	*P*
Groups 1 and 2	7.6	3.72	
1 and 3	7.9	4.56	<0.001
1 and 4	11.4	7.21	
1 and 5 a....	13.8	6.77	
3 and 5 a....	5.9	2.61	<0.05

It is evident, to judge from the average intrinsic viscosity, that hyaluronic acid is more or less 'degraded' in all groups except the recent traumatic effusions (1a), the average value here approximating closely to the value found for normal or probably normal fluids from the human knee joint (see chapter VII, page 176). Compared with the control group of normal synovial fluids of all types (table 19, page 177), the hyaluronic acid was also of somewhat lower intrinsic viscosity in the traumatic group 1a, the difference between the averages (7.2 ± 2.2) being significant. No significant difference was found between the sub-groups of groups 1 and 3. In group 5, however, the cases with systemic reactions (fever and/or increased sedimentation rate) showed a significantly lower average intrinsic viscosity than the

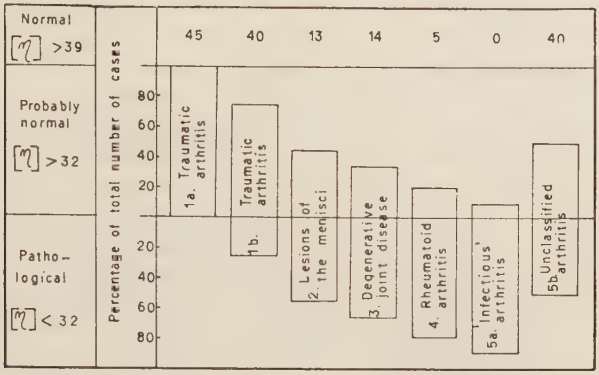

Fig. 26. Diagram illustrating diagnostic value of intrinsic viscosity test.

unclassified cases of sub-group 5b (see table 23). Although the changes in the state of the polysaccharide were evidently most marked in the fluids from rheumatoid and 'infectious' arthritis (groups 4 and 5a) the average intrinsic viscosities in these groups were not significantly lower than those observed in groups 2 and 3, i. e. lesions of the menisci, and degenerative joint disease. A difference of 5.9 ± 2.3 was probably present between the averages of groups 3 and 5a.

The possible value of the intrinsic viscosity as a diagnostic aid is illustrated by fig. 26.

Bearing in mind the small size of the series, the levels of intrinsic viscosity chosen to indicate 'normal' and 'pathological' values (> 39 and < 32, respectively) should be regarded as only provisional.

It follows that the test may be of value in detecting underlying joint disease in cases where an injury to the joint has been supposed to be the sole cause of the joint disability. The converse, however, is not true, since normal values of $[\eta]$ may be encountered in all types of fluids, although rarely in rheumatoid or infectious arthritis.

Apart from the unclassified group, 5b, the variation was most marked in degenerative arthritis (see standard deviations, table 23). Although the numbers were too small to allow of any definite conclusions, the diagrams in fig. 25 suggested heterogeneity in this respect in groups 2 and 5b.

DISCUSSION. DIAGNOSTIC VALUE OF TESTS

The more important chemical differences between fluids from different groups of arthritis are summarized in tabular form below. Special regard has been given to the possible diagnostic value of the various tests.

A comparison of the results summarized above with those of previous

Table 24. Diagnostic value of synovial fluid findings.

For definition of groups see pages 181—184.

Test	Summary of results	Diagnostic value of test
Protein concentration, g. per 100 ml.	Averages between 3.7 and 4.5 in groups 1–3 (traumatic arthritis and degenerative joint disease). Highly increased in rheumatoid and 'infectious' arthritis, averaging 5.1–5.5. Normal values, i.e. below 3.0, are found in traumatic fluids of small volume (below 10 ml.) and occasionally in the other groups.	Limited because of great variation. Levels below 4.0 are only occasionally found in rheumatoid and 'infectious' arthritis. Values above 6.0 are rare in traumatic arthritis and degenerative joint disease. In cases of small fluid volume, i.e. 10 ml. or less, a finding of more than 4.0 g. per 100 ml. in a 'traumatic' fluid is some indication that the injury is not the sole cause of the arthritis.
Hyaluronic acid concentration, g. per 100 ml.	In traumatic arthritis the concentration varies approximately in inverse proportion to the fluid volume. Values within the normal range for the human knee joint, i.e. 0.2–0.4, may be found in rheumatoid and 'infectious' arthritis and in degenerative joint disease, in spite of large fluid volumes. In comparable degrees of effusion lower values are found in traumatic arthritis than in the other groups.	Actual concentration is of no diagnostic aid in most instances unless related to the viscosity, or if the fluid volume is also taken into consideration (see below).
Total hyaluronic acid content of the fluid, mg.	On an average about 25 mg. is contained in the fluids of traumatic arthritis, compared with about 50 in degenerative joint disease and 75–100 in rheumatoid and 'infectious' arthritis. May exceed 300 in the latter groups.	Levels higher than 50 mg. are only occasionally found in traumatic disorders, compared with about 35 % of the cases in degenerative joint disease and 71–74 % in rheumatoid and 'infectious' arthritis.

Table 24 (cont.)

Test	Summary of results	Diagnostic value of test
Relative viscosity at 37° C.[1]	Varies greatly (averages between 6 and 44), as it reflects both quantitative and qualitative changes in the hyaluronic acid. Low values are commonest in rheumatoid and 'infectious' arthritis.	Of very limited value because of the great variation within all groups. Low viscosities are of no aid in differentiating between the groups. High values, i.e. more than 20, are only exceptionally found in rheumatoid or 'infectious' arthritis in contrast to higher levels in 30–60 % of the cases in groups 1–3 of traumatic and degenerative arthritis.
Intrinsic viscosity, calculated from the viscosity/concentration ratio, and used as a test for qualitative polysaccharide changes.	Usually normal values (above about 40) in traumatic arthritis. Averages, ranging from 37.4 to 51.5 in the normal fluids and group 1. Moderately below normal in traumatic arthritis associated with internal derangements of cartilage, and in degenerative joint disease, with averages about 30. Marked changes in rheumatoid or 'infectious' arthritis, averaging 26.3 and 23.9 in groups 4 and 5a. Normal values are found relatively frequently in degenerative joint disease and occasionally also in cases of inactive rheumatoid arthritis.	Values below about 32 are not found in normal fluids or those of uncomplicated traumatic arthritis, in contrast with 53 % of cases of traumatic arthritis associated with meniscus lesions[2], 64 % in degenerative joint disease, and 80–90 % in rheumatoid or 'infectious' arthritis. The test is thus of special value in detecting underlying joint disease in cases of supposed traumatic arthritis. The converse is, however, not true. Values below 20 are frequent only in acute arthritis with systemic reactions (about 35 % of the cases in group 5a), but may also be found in rheumatoid arthritis and degenerative joint disease (15 and 14 % respectively in groups 4 and 3). The test is of no value in differentiating between the two latter types of joint disease.

[1] As the viscosity of concentrated solutions depends greatly on the dimensions of the capillary used in the determination, the limits of variation given are only valid for the methods used in this investigation.

[2] Marked changes are present only in fluids aspirated within a few weeks of the actual injury (see table 33, page 208). In 5 cases in group 1b values of intrinsic viscosity below 32 were also found. These cases showed, however, signs of intra-articular cartilage lesions, although this diagnosis could not be confirmed.

authors is only possible to a limited extent. Bauer and associates, however, who have by far the greatest experience of this kind of analysis, report findings with regard to protein content of joint fluids which are essentially the same as those of this investigation (see for example Ropes, 1952). The values for relative viscosity are also comparable. On the other hand, no close relationship is present between the values for mucin concentration reported by these and other authors, and those of hyaluronic acid concentration reported here. No doubt this should be ascribed, at least in part, to the varying chemical composition of the mucin of pathological fluids (see Ropes *et al.*, 1947).

It appears that discrepancies must further exist between the results of the intrinsic viscosity test and the qualitative test for degradation of the synovial polysaccharide (see introduction, page 117). Thus in degenerative joint disease Ropes *et al.* (1947), using the precipitation test, found essential absence of mucin changes, but marked changes in most cases of rheumatoid arthritis. By the intrinsic viscosity test no gross differences can be found between the fluids of these types of arthritis. Since the two tests probably reflect the same type of hyaluronic acid changes, a possible explanation of the discrepancy may be that the result of the precipitation test also depends on the type and amount of proteins present.

Ragan and Meyer (1949) give much the same values for hyaluronic acid concentration in rheumatoid arthritis fluids as those of the present study. The index of polymerization used by these authors, i. e. the quotient between log viscosity and concentration, is not ideally suited for such comparisons, as it varies with concentration. Approximate calculations of intrinsic viscosities from the analytical data given showed, however, a similar range of values as that given for rheumatoid arthritis fluids in this paper, in spite of the different methods.

Determinations of hyaluronic acid concentration in synovial fluids have recently been made by Rössing (1952), who found high values in post mortem fluids from cases of rheumatoid arthritis and various inflammatory conditions. Numerous workers have determined relative viscosity, mucin, and proteins, in small series of pathological fluids. For results and discussions on the diagnostic value of these tests reference must be made to Kling (1938), Ropes *et al.* (1947), and Ropes (1952).[1]

FINDINGS IN FLUIDS FROM MISCELLANEOUS TYPES OF JOINT DISTURBANCE

The results of analysis in a small group of arthritic conditions closely related to rheumatoid arthritis, and a few cases of rheumatic fever,

[1] A monograph on synovial fluid by Ropes and Bauer (1953) has just appeared. It contains a detailed bibliography.

Table 25. Clinical types and chemical findings in fluids from arthritic conditions allied to rheumatoid arthritis (group 6).

The average hyaluronic acid and protein concentrations are given, and also the range and average of the values for intrinsic viscosity. Numbers in parentheses indicate numbers of protein determinations.

Type of arthritis	No. of cases	Hyaluronic acid, g. per 100 ml.	Total protein, g. per 100 ml.	Intrinsic viscosity		Remarks
				Range	Av.	
1. Subacute polyarthritis–rheumatoid arthritis?	2	0.116	5.3 (1)	21.0—32.2	26.6	These were two cases in which the diagnosis of rheumatoid arthritis was questioned.
2. Subacute polyarthritis following rheumatic fever	2	0.120	5.7 (2)	20.5—22.0	20.3	One of the cases was a relapse of rheumatic fever, the actual diagnosis given being 'polyarthritis acuta recidivans'.
3. Subacute polyarthritis associated with urethritis and conjunctivitis.— Reiter's syndrome?	3	0.123	5.3 (2)	17.0—40.9	25.3	All patients presented some degree of fever, high sedimentation rate, negative tests for gonorrhea, and no signs of cardiac involvement.
4. Ankylosing spondylitis						Disease in active stage with high sedimentation rate. The fluid of 4 b was obtained from an unaffected knee joint, volume of fluid 1.5 ml.
a) With bilateral involvement of the knee joint	1	0.205	7.4		35.0	
b) Without evidence of peripheral joint involvement	1	0.398	2.5		34.4	
5. Acute disseminated lupus erythematosus?	1	0.151	4.7		23.2	Post mortem diagnosis 4 months later.
6. Psoriatic arthritis	1	0.048	4.8		41.3	Chronic involvement of mainly knees and toes. Low sedimentation rate.

are shown in table 25, where some essential clinical data are also given.

These cases were separated from group 4 (rheumatoid arthritis) for theoretical reasons, but it is evident that in spite of the great variation the findings are much the same as in that group. The average values for the 10 pathological fluids in the group (total protein 5.5 g. per 100 ml., hyaluronic acid 0.124 g. per 100 ml., and intrinsic viscosity 27.1) did not thus differ significantly from the corresponding values in the rheumatoid arthritis group. The relative viscosities were also low (below 11) in all cases except for two of ankylosing spondylitis, in which the values were 70 and over 300 respectively (cases 4a and 4b, see the table). On subsequent aspiration some months later the fluid in the former case showed the same discrepancy between the highly increased protein concentration and the essential absence of hyaluronic acid changes. The fluids from 2 cases of subacute arthritis following rheumatic fever showed, on the other hand, a degree of change comparable to that usually found in acute febrile arthritis regardless of the aetiology, which is worth mentioning since the fluids in rheumatic fever have been reported to resemble fluids of traumatic type (see Ropes *et al.*, 1947).

The findings in fluids from a group of miscellaneous types of joint disturbance associated with some trauma to the joint are shown in table 26. The group consisted partly of cases excluded from the traumatic arthritis groups of the main series for various reasons. If the criteria for the grouping of the series had been slightly different, some of the cases might well have been included among the traumatic groups. A separate discussion of the fluids of group 7 seemed, however, desirable, since the results served both to illustrate the possible diagnostic value of the intrinsic viscosity test, and to elucidate to some extent the mechanism of the hyaluronic acid changes indicated by this test.

The table shows that with regard to the values of total protein and hyaluronic acid concentrations the fluid findings were much the same as those in group 1 (see tables 21 and 22, pages 184 and 186), except for the cases in sub-group 5. The main differences were found in connexion with intrinsic viscosity, see below.

Sub-group 1 (see table 26) consisted of cases of effusions appearing usually about one month after operations on the knee joint (performed for various conditions). Of the 7 cases, 4 were lesions of the semilunar cartilages, one of osteochondritis dissecans, one of probable tuberculous bursitis of the popliteal fossa, and one of penetrating injury. In no instance was there any evidence of bacterial complication, the appearance of an effusion being associated in most cases with strain on resumption of joint function. The radiograms revealed varying degrees of degenerative joint change in 3 cases.

The explanation of the marked changes in the state of the hyaluronic acid in these fluids was not clear. The average intrinsic viscosity of 19.1 was even lower

Table 26. Findings in fluids from miscellaneous types of joint disturbance associated with trauma (group 7).

The values given are the arithmetic mean, and the range, of intrinsic viscosity. Numbers in parentheses indicate numbers of protein determinations.

Type of joint disturbance	No. of cases	Hyaluronic acid, g. per 100 ml.	Total protein, g. per 100 ml.	Intrinsic viscosity	
				Range	Average
1. Synovitis after operations on the knee joint	7	0.119	3.9 (4)	12.1—28.8	19.2
2. Synovitis after arthrography	2	0.080	5.0 (1)	17.0—30.0	23.5
3. Joint disturbance secondary to previous severe injury	4	0.128	3.6 (3)	25.0—34.8	31.0
4. Osteochondritis dissecans	1	0.113	4.7		36.1
5. Cases of 'traumatic arthritis' excluded from group 1, page 181, for various reasons (see text)	4	0.173	4.3 (4)	18.3—40.6	27.4

than the corresponding value in acute febrile arthritis, but the hyaluronic acid concentration was notably low. The latter finding was in obvious disagreement with the conditions in the other groups, where the degree of 'degradation' of the hyaluronic acid was roughly paralleled by an increase in the concentration of the polysaccharide (see below, table 40, fig. 34). Possibly this may be an indication that different mechanisms are responsible for the change in the hyaluronic acid.

Sub-group 2 included 2 fluids aspirated 2 days after the joint had been radiographed for suspected lesions of the menisci. In one of the cases ordinary air had been used, and here there were no signs of synovitis. The sterile fluid of the second case also showed marked changes in the hyaluronic acid, in spite of the short duration of the synovitis and the fact that the arthrogram revealed normal conditions. In the former case the healthy knee joint was simultaneously aspirated, 1/2 ml. fluid with normal analytical findings being obtained (see table 19, page 177).

Sub-group 3 consisted of 3 cases where the disability was apparently related to internal derangements of the knee joint of 2—18 years duration. The remaining case had a history of recurring effusion on unaccustomed exercise, following a sprain 15 years earlier. Repeated aspirations had been performed in two of the cases before the taking of the samples analysed. The findings in this group, and in the case of osteochondritis dissecans, were similar to those in traumatic arthritis, except for slightly lowered intrinsic viscosity.

Sub-group 5 consisted of 3 cases which had been excluded from group 1 of the main series, as signs of multiple joint involvement were present at the time of aspiration, or appeared shortly afterwards. The clinical diagnoses given were 'contusio gen. + polyarthritis?', 'hydrops traumatica + polyarthritis?', and 'status post distorsio gen. + myalgiae + arthralgiae'. High cell-counts were found in 2 of the fluids, and an increased sedimentation rate in one. The findings were those

of traumatic effusions, except for decreased intrinsic viscosity in 2 cases. The remaining case in sub-group 5 had persistent synovitis with moderately increased sedimentation rate. The relation of this to an insignificant contusion which was reported seemed dubious. Some weeks before the sample was taken a large, somewhat cloudy effusion without signs of blood admixture had been withdrawn from the joint.

Finally, the results of analysis of a few bursa fluids deserve some mention, the changes in the fluids being much the same as could be expected in the synovial fluids from the respective joint disorders. Thus the findings in a fluid taken from the prepatellar bursa in a case of rheumatoid arthritis were in keeping with those found in samples of knee joint effusions from the same patient. A fluid obtained from a patient with chronic tendovaginitis of the extensor sheaths of the wrists complicating a rheumatoid arthritis which was otherwise quiescent, showed a hyaluronic acid concentration of 0.185 g. per 100 ml. and an intrinsic viscosity of 32.4, the fluid volume being 4 ml. A sample taken from a bursal sac which had developed round the nail in a case of internally fixated fracture of the neck of the femur was also analysed. 10 ml. of fluid was obtained on re-operation, which contained 0.050 g. hyaluronic acid per 100 ml. and had an intrinsic viscosity of 35.2.

STABILITY OF THE VISCOSITY AT 37° C

In confirming the results of previous authors (Robertson *et al.*, 1940; Ropes *et al.*, 1947; Meyer, 1947) it was not possible to demonstrate the presence of hyaluronic-acid-depolymerizing agents in the pathological fluids. There was no appreciable fall in viscosity on incubation at 37° C. even if continued for several days unless the fluids had been contaminated with bacteria, as sometimes occurred in the animal samples. In these cases other signs of bacterial decomposition were observed later. If stored at — 18° C. the fluids retained their viscosity practically unchanged even for months.

In connexion with these studies it was however observed that prolonged incubation at 37° C. was accompanied by a usually slight but sometimes considerable increase in viscosity in some of the fluids, but no measurable changes occurred if the fluids were kept at room-temperature. Since no reports on this phenomenon could be found in the literature a closer study seemed warranted, especially with regard to the observation made early in the investigation of an increase in relative viscosity of more than 100 % in a sample from a case of osteochondritis dissecans (see fig. 28, page 200). In the routine determinations of relative viscosity the stability of the viscosity at 37° C. was therefore tested over a period of about 20 hours in most of the

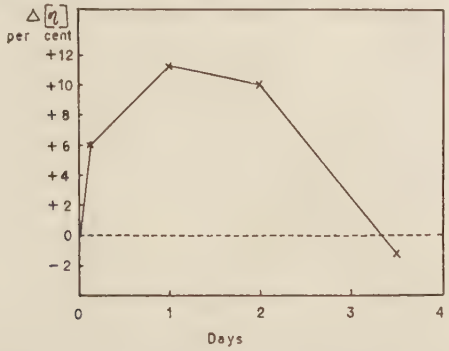

Fig. 27. Changes in viscosity on incubation of synovial fluid at 37° C.

The average percentage changes in intrinsic viscosity, $\Delta[\eta]$, are given for a group of 6 fluids from traumatic arthritis.

fluids. Between determinations the fluid was left in the viscometer, which was kept sealed to prevent evaporation.

Some observations on the general type of change will be described, before entering into the results obtained in the different groups.

Influence of incubation-time. The changes in viscosity occurring on prolonged incubation at 37° C. are shown in the diagram in fig. 27 for 6 fluids in which the increase in viscosity was marked. Since the fluids had very different relative viscosities the corresponding values of intrinsic viscosity have been calculated, and the averages for the 6 samples given.

The changes are evidently reversible in that the initial level of viscosity is again reached after about 3—4 days. In some cases the end-values were slightly lower than the initial ones, the decrease approximately corresponding to that observed when the fluids were stored at room-temperature or at + 4° C. over the same period of time.

The effect of varying the temperature is shown in fig. 28.

It can be seen that the increase in viscosity was more marked at + 40° C. than at + 37° C. In the former case the test sample was kept in an ordinary glass tube and the phenomenon could thus not be ascribed to protein alterations, such as denaturation or film formation, which might have resulted from storage and handling of the sample in the viscometer. Fig. 28 shows further that if incubation at + 37° C. is interrupted and the fluid returned to + 4° C. there is no rapid fall in viscosity such as that observed after some days at 37° C., but only a gradual decrease.

Relation of viscosity changes to the initial relative viscosity. The increase in relative viscosity was found to be proportional to the initial viscosity of the sample. Thus a rise of η_{rel} of up to about 30 was ob-

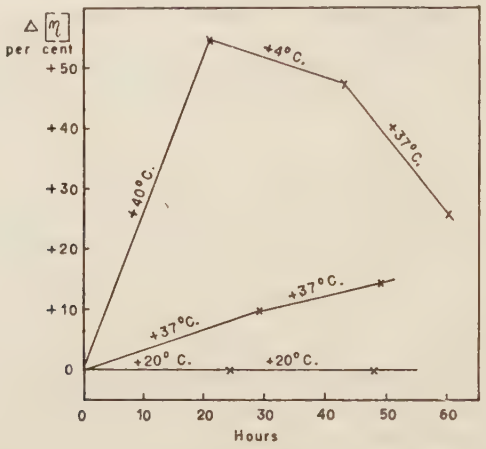

Fig. 28. Changes in viscosity of a fluid from osteochondritis dissecans on incubation at different temperatures.

The percentage changes in intrinsic viscosity, $\Delta[\eta]$, are given.

served in some fluids of high viscosity, whereas the changes occurring in fluids of low viscosity were always comparatively slight. If, however, the changes were expressed in terms of intrinsic viscosity, there was no obvious relationship between the degree of changes and the relative viscosity of the sample. The change in intrinsic viscosity occurring on incubating the undiluted fluid at 37° C. for about 20 hours was chosen as a basis of comparison, and denoted as $\Delta[\eta]$.

The effect of diluting the fluids is shown in table 27. In a series of synovial fluids comparative determinations of $\Delta[\eta]$ were made on the undiluted fluids and on the same samples diluted with phosphate-NaCl of pH 7.0. The changes, as measured in terms of relative viscosity, were considerably less marked in the diluted fluids, but as is evident from table 27, the average value of $\Delta[\eta]$ was much the same in the 1:1 dilutions as in the undiluted fluids. At higher degrees of dilution the changes were on the other hand comparatively slight.

Similar results were obtained if dilutions were made with 0.2 M-Nacl only.

The results given in table 28 show that there was a significant increase in average intrinsic viscosity in the fluids of the traumatic and degenerative groups, in contrast with the findings in the rheumatoid or infectious types of fluids. In the latter type of fluids the values were often slightly lower after 20 hours incubation at 37° C. than the initial ones.

The variation was, however, great in all groups. However this can probably be ascribed in part to the fact that the samples had been stored

Table 27. Influence of diluting the test sample on the viscosity changes at 37° C.

Dilutions were made with phosphate-NaCl, pH 7.0. The viscosity changes occurring in the samples on incubation at 37° C. for about 20 hours were determined.

Series	Average increase in intrinsic viscosity %
I. (8 fluids)	
Undiluted	4.6
Diluted 1 : 1	5.6
II. (10 fluids)	
Undiluted	3.4
Diluted 1 : 2—4	0.8

Table 28. Stability of the viscosity of synovial fluids at 37° C.

The changes in viscosity occurring on incubation of the undiluted fluids at 37° C. for about 20 hours were determined. For definition of groups, see pages 181 and 197. P is the probability that the changes are due to random variation. $M[\eta]_0$ = average of initial intrinsic viscosity.

Group	Number of fluids	Changes in intrinsic viscosity at 37° C., 20 hrs.		
		$M \pm \varepsilon (M)$	P	M as per cent of $M[\eta]_0$
1. Traumatic arthritis	31	$+1.48 \pm 0.51$	<0.01	+3.9
2. Lesions of the menisci	11	$+0.50 \pm 0.55$		
3. Degenerative joint disease	33	$+0.44 \pm 0.13$	<0.01	+1.5
4. Rheumatoid arthritis	39	0.00 ± 0.09		
5 a. Miscellaneous forms of arthritis with systemic reactions	16	-0.04 ± 0.31		
7. Miscellaneous forms of joint disturbance associated with trauma to the joint	18	$+0.96 \pm 0.35$	<0.05	+3.9

at — 18° C. for various periods of time before the determinations, since a few experiments indicated that the viscosity phenomenon decreased on storing.

The table further shows that in the fluids of group 7 (see table 26,

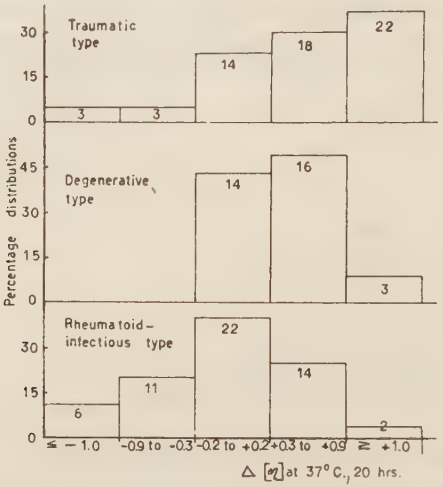

Fig. 29. Distributions of cases of traumatic type (groups 1, 2, and 7), degenerative type (group 3), and rheumatoid-infectious type (groups 4 and 5a), according to viscosity changes in the fluids at 37° C.

For definition of the groups, see page 181. $\Delta[\eta]$ = change in intrinsic viscosity on incubation of the undiluted sample at 37° C. for about 20 hrs. The class limits of $\Delta[\eta]$ were arbitrarily chosen, as shown in the fig. The numerals in the columns indicate number of cases.

page 197), i. e. cases where the joint disturbance was associated with some type of trauma, the viscosity changes were similar to those found in the fluids of traumatic arthritis. Since the average intrinsic viscosity in group 7 was only 25.1 against 38.1 for the fluids of group 1, it appeared that the viscosity phenomenon was independent of the degree of polymerization of the synovial polysaccharide. This was also evidenced by the fact that in the normal fluids the viscosity was invariably lower after 20 hours at 37° C., the average decrease in intrinsic viscosity of 14 cases being about 5 %. This value cannot, however, be compared with those in table 28, since the determinations were usually made on highly diluted samples.

The distribution diagrams given in fig. 29 indicate that this simple test may be of some aid in differentiating fluids of traumatic disorders or degenerative joint disease from those of rheumatoid and infectious arthritis.

DISCUSSION

The above results all seem consistent with the view that the changes in viscosity described are intimately associated with the incidence of injury to the joints. Further, as the changes were mostly such that

they could not be accounted for by alterations in the fluid proteins, the increase in viscosity should reflect some sort of change in the state of the hyaluronic acid or possibly polysaccharide-protein complex. Moreover, the alterations bore no obvious relation to concentration or intrinsic viscosity of the synovial polysaccharide, which would repudiate the possible explanation that the phenomenon might be ascribed to variations in degree of molecular aggregation. It seems also unlikely that the increase in viscosity can be ascribed to slight changes in pH.

The absence of measurable changes at room temperature or in the cold, in addition to the higher rate of the viscosity increase observed at 40° C. compared with 37° C., suggest in fact an enzymatic type of reaction. It is tempting to presume that the viscosity changes in the traumatic fluids might reflect a polymerization process otherwise restricted to the periarticular tissue. Bearing in mind the size of the hyaluronic acid molecule, the final steps at least of the synthesis probably take place extracellularly, and accordingly the active agents might well be released in increased amounts into the joint cavity in connexion with an injury to the joint. In keeping with this was the fact that viscosity changes were most marked in fluids of large total volume which had been aspirated in immediate relation to a significant trauma. The average increase in intrinsic viscosity was thus 5.1 % in group 1a compared with 1.9 % in group 1b. In the latter group the fluids had usually been collected some time after the actual injury, and the degree of effusion was nearly always slight.

The findings in the degenerative arthritis group do not support the suggestion that the causative factor is simply bleeding into the joint cavity.

In a fluid from osteochondritis dissecans, which showed a 50 % increase in η_{rel} on storage for 20 hours at 37° C., the degradation curves after addition of a constant amount of hyaluronidase were compared in untreated and incubated specimens. The fact that the degradation rates were practically identical, 0.48 and 0.50 VU (see page 162) respectively, seemed to support the view that the viscosity changes might be ascribed to a polymerization, probably of enzymatic nature, of the synovial polysaccharide.

CHAPTER IX

Relation of the Changes in the Fluids to Certain Factors

The great variation of most chemical findings within all groups of joint fluids is shown in table 29.

A comparison with the errors of the analytical procedures shows that these could only account for a comparatively small fraction of the variation. Table 30 further gives some idea of the individual variability as calculated from the results of repeated analysis of fluid from the same patients. It should be noted that in several cases the

Table 29. Variability of analytical findings in normal and pathological synovial fluids.

For definition of groups, see page 181. σ = standard deviation, V = standard deviation expressed in percentage of the mean.

Group	Hyaluronic acid, g. per 100 ml.			Intrinsic viscosity			Total protein, g. per 100 ml.		
	No.	σ	V	No.	σ	V	No.	σ	V
Normal fluids	26	0.114	72.6	26	9.7	21.0	17	1.0	46.0
Pathological fluids									
1 a. Traumatic	20	0.038	37.2	20	5.0	12.8	17	1.3	30.8
1 b. arthritis	20	0.075	42.7	20	7.4	20.4	13	1.3	34.4
2. Lesions of the menisci	15	0.063	35.2	15	6.8	22.6	11	1.6	39.2
3. Degenerative joint disease	42	0.066	47.1	42	9.0	30.2	22	1.1	26.0
4. Rheumatoid arthritis	40	0.061	40.1	40	7.8	29.4	33	1.0	18.5
5 a. 'Infectious' arthritis	20	0.066	49.2	20	7.9	33.2	11	1.0	18.8
5 b. Unclassified arthritis	20	0.059	43.0	20	9.3	27.9	13	1.6	37.2

Table 30. *Individual variability of findings in fluids from miscellaneous types of arthritic disease.*

Since the variation between fluids from both knees appeared to be the same as that between fluids obtained by repeated aspiration of the same knee, the individual variability, s, for a certain patient was obtained in the usual way from all determinations. The total estimate of the individual variability, σ_i, was then calculated from the formula

$$\sigma_i = \pm \sqrt{\frac{\Sigma f s^2}{\Sigma f}},$$

where f is the number of degrees of freedom corresponding to the value of s.

Test	Number of fluids	σ_i	σ_i as per cent of mean
Hyaluronic acid, g. per 100 ml.	42	0.042	32.4
Total protein, g. per 100 ml.	22	0.8	15.7
Relative viscosity	42	16	110.7
Intrinsic viscosity	42	4.9	16.7

time interval between the aspirations was considerable, the samples compared having been taken at different stages of the disease.

The results will be presented of some attempts to analyse the sources of variation of the fluid changes in the types of arthritic disease studied. Since the most important differences between the groups of fluids were found by means of the intrinsic viscosity test, the relationship between this test and various clinical features will be described.

The results of repeated tests in the same patient were included in some instances, where the time interval between the aspirations was considerable, or where other significant differences between the samples with regard to the factor studied were present.

The influence of the following factors was studied in all groups. 1. Sex and age. 2. Duration of disease. 3. Degree of effusion. Brief accounts of the relationship of the tests to the degree of systemic reactions, X-ray findings, extent of joint involvement, and the number of white cells present in the fluids, are also given for part of the series.

SEX AND AGE

The influence of sex- and age-factors is shown in table 31.

In spite of the small numbers it seems justified to conclude that the differences in intrinsic viscosity between the groups of fluids (see table 23, page 189) could not reasonably be due to sex and age.

Table 31. Relation of intrinsic viscosity to sex and age.

For definition of groups see page 181.

Group	Intrinsic viscosity						Age group, years	Intrinsic viscosity		
	Men [1]			Women [1]						
	No.	$M \pm \varepsilon(M)$	σ	No.	$M \pm \varepsilon(M)$	σ		No.	$M \pm \varepsilon(M)$	σ
1. Traumatic arthritis	29	38.0 ± 1.2	6.3	11	37.0 ± 1.7	5.5	5—30	18	37.7 ± 2.0	8.4
							31—64	18	37.2 ± 0.7	3.2
3. Degenerative joint disease	22	27.5 ± 2.1	10.1	20	32.4 ± 1.7	7.8	27—52	21	29.5 ± 1.6	7.6
							53—82	21	30.1 ± 2.3	10.4
4. Rheumatoid arthritis	21	27.2 ± 2.1	9.4	19	25.3 ± 1.3	5.5	8—41	19	26.5 ± 1.9	8.4
							42—68	20	26.0 ± 1.7	7.4
5. Arthritis of infectious or unknown aetiology	24	28.3 ± 1.8	8.7	16	28.9 ± 2.9	11.5	17—37	20	31.4 ± 2.2	9.9
							40—64	20	25.7 ± 2.0	9.1

[1] The distribution of the cases according to age was essentially even between these sub-groups.

Some of the data deserve special mention. The essentially similar averages of intrinsic viscosity in the two age groups of both the degenerative and the traumatic arthritis groups may be taken as some indication that the hyaluronic acid changes do not depend entirely upon the degree of degenerative change but are related to other factors (see also chapter X).

The standard deviations possibly indicate differences in variation between the sub-groups in some cases, e. g. between the sexes in rheumatoid arthritis (difference of $\sigma = 3.9 \pm 1.7$) and between the age groups in the traumatic arthritis group (difference of $\sigma = 5.2 \pm 1.5$). The significance of these findings could however not be established on the basis of the present series.

Only insignificant differences were found between the sub-groups of table 31 in comparing the results of the other chemical tests.

DURATION OF DISEASE

The relation of the intrinsic viscosity to the duration of disease was analysed by subdivision of each group according to this factor, as shown in table 32.

The values for duration refer to the conditions in the aspirated knee joint, except that in the rheumatoid arthritis group the duration

Table 32. Relation of intrinsic viscosity to duration of disease.

For definition of groups, see page 181. The groups were subdivided according to the duration of joint disability as further explained in the text, the approximate median values in the sub-groups being given.

Group	Duration of joint disability. Median	No.	Intrinsic viscosity	
			$M \pm \varepsilon (M)$	Difference of M between sub-groups $D \pm \varepsilon (D)$
1. Traumatic arthritis	4 days	20	37.3 ± 1.6	0.9 ± 2.1
	2 months	20	38.2 ± 1.3	
2. Lesions of the menisci[1]	1 week	8	25.7 ± 2.1	9.4 ± 2.4
	2 months	8	35.1 ± 1.3	$P < 0.01$
3. Degenerative joint disease	1 month	19	28.1 ± 2.0	5.6 ± 2.9
	2 years	19	33.7 ± 2.1	
4. Rheumatoid arthritis (duration of disease)[2]	2 years	19	26.9 ± 1.8	0.6 ± 2.5
	10 years	19	26.3 ± 1.8	
4. Rheumatoid arthritis (duration of knee joint disability)	3 months	15	26.8 ± 2.0	1.3 ± 3.1
	2 years	15	25.5 ± 2.3	
5. Arthritis of infectious or unknown aetiology	1 week	20	28.6 ± 2.3	0.2 ± 3.1
	1 year	20	28.4 ± 2.0	

[1] One instance of analysis repeated about 3 months after the initial injury is included, and further illustrates the results given in the table. This fluid showed an intrinsic viscosity of 35.4 against 22.0 in the fluid collected about 2 weeks after injury. On operation one week after the second aspiration a rupture of the lateral meniscus was found. A histopathological diagnosis of 'fibrinoid degeneration in menisci, mild chronic inflammatory changes in synovial membrane' was made.

[2] Reliable information about the time of onset of knee joint disability was only available in a limited number of cases.

of the actual disease regardless of the time of onset of knee joint disability was also taken into consideration.

In the chronic conditions the total duration of disease, i. e. not only that of an acute relapse, was chosen as a basis of comparison.[1]

Since reliable data were not always available, groups 1—5 are not quite identical with the corresponding groups of chapter VIII.

As shown by the table, there was no difference in average intrinsic viscosity between the sub-groups of the traumatic arthritis group. The sub-groups were approximately the same as those of 1a and 1b (see page 181). In group 2 of traumatic arthritis associated with lesions of

[1] Subdivision according to the probable duration of the actual effusion was also attempted in some groups, the results being about the same as those of table 32.

the semilunar cartilages, a significant difference was on the other hand found between the sub-groups, although the number of cases was small. In the degenerative arthritis group, too, there was a higher average intrinsic viscosity in the sub-group of long duration, but the difference was not significant. The latter results thus did not indicate any close relationship between the degree of hyaluronic acid change and the severity of degenerative arthritis, in accordance with other results pointing in the same direction.

Only insignificant differences in intrinsic viscosity were found on sub-division of groups 4 and 5 of rheumatoid and unclassified arthritis. The results were the same in the latter group, if the cases of infectious arthritis of known aetiology were excluded.

Bearing in mind the significant difference in intrinsic viscosity found between the sub-groups 5a and 5b (see table 23, page 189) it should be noted that the distribution of the cases according to sedimentation rate was approximately even between the 'acute' and the 'chronic' sub-groups of group 5.

Although the data are presented with circumspection since the numbers in the sub-groups were small and the clinical data probably often unreliable, it is concluded that the changes in the state of the hyaluronic acid were as a rule unrelated to the duration of disease. Neither could any of the results of the other chemical tests be related to this factor.

The marked difference between the sub-groups of group 2 (lesions of the menisci) was one exception to the above statement. Thus the

Table 33. Relation of intrinsic viscosity to interval between injury and aspiration in some forms of arthritis associated with trauma to the joint.

For definition of groups, see page 181. The subdivision of the groups is further explained in the text. The average intrinsic viscosities are given in the various sub-groups.

Group	Interval between injury and aspiration			
	Less than 1 month		More than 1 month	
	No.	Intrinsic viscosity	No.	Intrinsic viscosity
1 b. Traumatic arthritis	8	30.2	13	38.6
2. Lesions of the menisci	8	25.7	8	35.1
3 a. Degenerative joint disease	9	27.9	9	33.4
Total	25	27.0±1.5	30	36.1±1.2

Difference between averages of main sub-groups 9.1±1.9 ($P<0.01$)

incidence of two peaks (diagram, fig. 25) could in fact be referred to the heterogeneity of the group with respect to the interval between injury and aspiration. As the findings apparently indicated that the hyaluronic acid changes might in some way be related to the event of damage to cartilage in the joint, it seemed of interest to study the conditions in other groups of arthritis associated with trauma to the joint. The results given in table 33 justify this, as to some extent also does the close similarity between the averages of intrinsic viscosity in the sub-groups of group 1a. Here the sample had usually been collected in immediate relation to an uncomplicated sprain, whereas the cases of group 1b had exhibited symptoms, such as locking, suggestive of internal derangement following acute sprains or repeated minor injuries. In the cases of degenerative joint disease (group 3a) where an injury had either precipitated the onset or aggravated the joint disability, intra-articular cartilage lesions might well also have occurred in connexion with the trauma.

The interpretation of this apparent relationship between the fluid changes and acute damage to the cartilage will be further discussed in chapter X.

DEGREE OF EFFUSION

The pathophysiology of effusion formation is probably very complex. The colloid balance between the fluid and the surrounding synovial tissue would, however, appear to be a factor of importance.

It seemed of interest to correlate the chemical fluid findings with the values of total volume, see table 22, page 186 (i. e. the amount of fluid which could be withdrawn from the joint), although these values can only be regarded as a very rough measure of the degree of effusion.

The results obtained for group 1 of the main series are illustrated by the dot diagrams in fig. 30.

The hyaluronic acid concentration evidently varies inversely as the degree of effusion in traumatic arthritis, whereas the concentration of total protein varies directly. The rank correlation coefficients were found to be significant in both instances (see table 34). The degree of correlation was however only moderately high, although due consideration should be taken to the relative inaccuracy of the volumes when judging the findings.

No relationship between intrinsic viscosity and fluid volume could on the other hand be demonstrated.

It follows (see chapter VIII, pages 185 and 187) that the degree of effusion must be taken into consideration when assessing the changes in total protein and hyaluronic acid concentration of fluids of supposed traumatic origin.

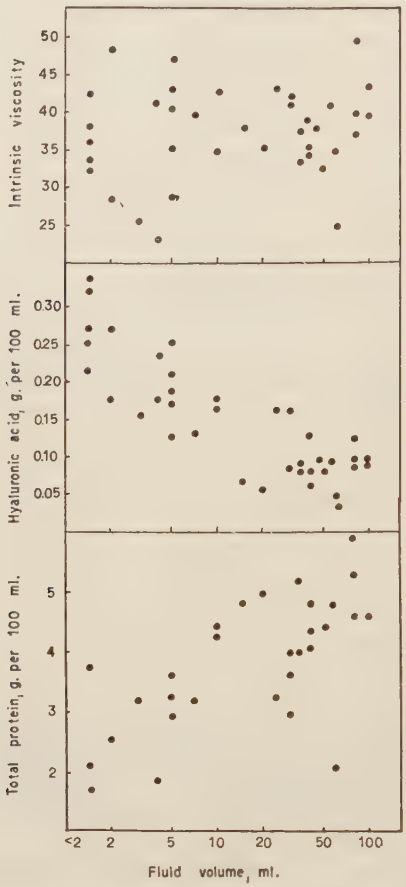

Fig. 30. Diagram showing the relation of the fluid findings to the log of fluid volume, in traumatic arthritis (group 1).

See also table 34.

Table 34 illustrates the conditions also for the groups 2—5.

To judge from the correlation coefficients given, no relationship between hyaluronic acid concentration and fluid volume was present in degenerative or rheumatoid arthritis, and the results were the same for the cases collected in group 5.[1]

[1] Insignificant correlation coefficients of + 0.17 and — 0.33 respectively were found in sub-groups 5a and 5b.

Table 34. Correlation between degree of effusion and chemical fluid findings in different types of arthritis.

For definition of groups, see page 181. The correlation coefficients, ϱ, calculated by the rank method, between the fluid volume and the chemical findings are given. P is the probability that the correlation might have arisen by chance.

Group	Correlation between fluid volume and–					
	Hyaluronic acid concentration		Total protein concentration		Intrinsic viscosity	
	No.	$\varrho \pm \varepsilon$ (ϱ)	No.	$\varrho \pm \varepsilon$ (ϱ)	No.	$\varrho \pm \varepsilon$ (ϱ)
1. Traumatic arthritis [1]	38	-0.79 ± 0.16 $P<0.001$	30	$+0.69 \pm 0.19$ $P<0.001$	38	$+0.13 \pm 0.16$
2. Lesions of the menisci	15	-0.57 ± 0.27 $P<0.05$	11	0.00 ± 0.32	15	$+0.01 \pm 0.27$
3. Degenerative joint disease	34	-0.16 ± 0.17	16	$+0.16 \pm 0.26$	34	-0.10 ± 0.17
4. Rheumatoid arthritis	34	-0.23 ± 0.17	31	$+0.03 \pm 0.18$	34	0.00 ± 0.17
5. Arthritis of infectious or unknown aetiology	38	-0.01 ± 0.16	23	$+0.68 \pm 0.21$ $P<0.01$	38	-0.27 ± 0.16

[1] Two samples of normal human fluids are included.

With regard to protein concentration, a correlation of about the same degree as in the traumatic group was also found in group 5 (see the table). To judge from the correlation coefficients this was of about the same degree in the sub-group 'arthritis with systemic reactions' (5a) as in the 'unclassified' sub-group (5b), the values being $+0.71 \pm 0.32$ and $+0.69 \pm 0.30$ respectively. Finally, in no group could any correlation between intrinsic viscosity and fluid volume be demonstrated.

DISCUSSION

The findings do not support the theory that changes in hyaluronic acid might be of importance in the disturbed water metabolism of the arthritic joint. This might otherwise have been suspected bearing in mind the high water-binding capacity of synovial mucin demonstrated by Ropes *et al.* (1939). These authors found the osmotic pressure effect per gram mucin to be nine times that of serum albumin, as estimated from the difference between the observed colloidal osmotic

pressure of normal cattle fluid and the pressure calculated from the protein content of the sample. An apparent relationship between the state of the mucopolysaccharides of connective tissue and its water content has further been demonstrated by several authors (see for references Ropes *et al.*, 1947; Kulonen, 1952).

Even complete enzymatic degradation of the polysaccharide component has been shown to influence only insignificantly the fluid osmotic pressure as calculated from the depression of the freezing point (Kulonen, 1951). In spite of this, the hyaluronic changes occurring in some types of pathological fluids may well be thought to affect also the water-binding capacity of the polysaccharide by alterations in the colloidal state. There was, however, no indication that this might be a factor of importance, with regard to the uniform lack of any correlation between the intrinsic viscosity and the fluid volume.

The inverse relationship between hyaluronic acid concentration and degree of effusion demonstrated in the 'traumatic' group can probably be explained as essentially due to dilution, i. e. the exudation occurring into the joint cavity is only accompanied by slight to moderate increase in hyaluronic acid 'production'. In other types of joint disturbance increased hyaluronic acid production usually parallels the degree of effusion (see discussion in chapter X, page 224).

The lack of parallelism between the release of proteins and the secretion of fluid into the joint cavity, demonstrated in the degenerative and rheumatoid arthritis groups, may be taken as some indication of the importance of such factors as impaired resorption in these chronic conditions. In the groups of arthritic disorders of essentially acute type there was on the other hand a comparatively high correlation between degree of effusion and the protein content of the fluids.

Finally, some notes on the relation of relative viscosity to fluid volume will be added. Although the η_{rel} of the undiluted fluids was usually not determined in samples of high viscosity, the values could be calculated according to eq. (5), page 140, from which it is evident that the η_{rel} is a function of the product of intrinsic viscosity and concentration (see fig. 20, page 178).

In the absence of qualitative changes of the hyaluronic acid the relative viscosity should vary as its concentration in the fluids. In accordance with this was the finding of a rank correlation coefficient between fluid volume and η_{rel} of -0.70 ± 0.16 ($P < 0.001$) for traumatic arthritis fluids (group 1, table 34) in close agreement with the coefficient for fluid volume and hyaluronic acid concentration of -0.79. For the groups 2—5 on the other hand only insignificant negative correlations were found between fluid volume and relative viscosity (range of coefficients -0.28 to -0.33). The relation of the averages of η_{rel}, obtained from the values of $[\eta] \cdot c$, to those of fluid volume for the subdivided groups is illustrated in fig. 31.

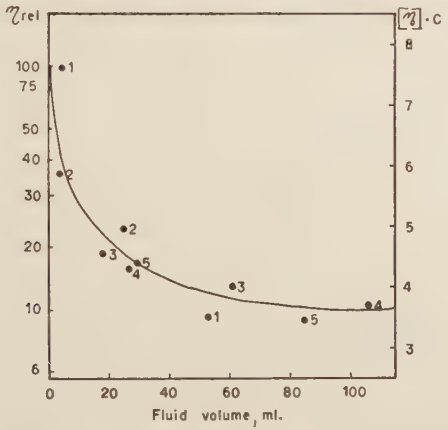

Fig. 31. Relation of fluid viscosity to fluid volume in groups 1–5.

The numerals indicate groups of arthritis, as defined in page 181. The groups were divided according to fluid volume, and the average fluid volumes plotted against average relative viscosities (left ordinate) for the sub-groups. The latter values were calculated from the averages of intrinsic viscosity and hyaluronic acid concentration ($[\eta] \cdot c$, right ordinate) according to equations (4) or (5). Rank correlation coefficient for the 10 couples $= -0.90 \pm 0.33$ ($P < 0.01$).

SYSTEMIC REACTIONS

As is evident from the results given in chapter VIII, the fluid findings were essentially different in the sub-groups obtained on arrangement of group 5 according to systemic reactions (fever and/or increased sedimentation rate). Thus in sub-group 5 a (miscellaneous forms of arthritis with systemic reactions) the average intrinsic viscosity was significantly lower than in the unclassified sub-group 5 b. Further, lower values for relative viscosity, and higher protein concentrations and fluid volumes, were found in the former group, whereas the hyaluronic acid concentration was about the same in both.

It seemed of interest to investigate whether a similar relationship between the degree of systemic reactions and the fluid findings could be demonstrated also in the group of rheumatoid arthritis, where the distribution of the cases with respect to degree of systemic reactions was much the same as in group 5.

The relation of the intrinsic viscosity test to the sedimentation rate (Westergren method) in the rheumatoid arthritis group is illustrated by the scatter diagram in fig. 32. The corresponding values in the cases of group 5 where the sedimentation rate was available are included for comparison.

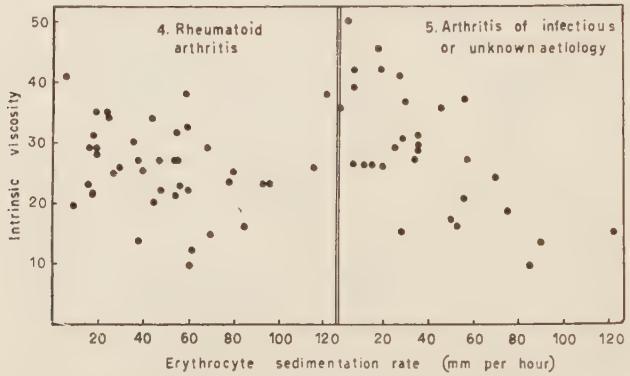

Fig. 32. Correlation between erythrocyte sedimentation rate and fluid intrinsic viscosity.

For definition of the groups, see page 182. By the rank method, a correlation coefficient of -0.27 ± 0.16 was found for the 40 cases of group 4, the corresponding value for the 30 cases of group 5 being -0.58 ± 0.19 ($P < 0.01$). A value of -0.48 ± 0.23 was found for the 20 cases of sub-group 5a. ($P < 0.05$.)

The actual sedimentation rates determined at the time of aspiration are given throughout. The results were essentially the same if the average value of 3 consecutive determinations were used instead of the above values in the rheumatoid group, where repeated tests had been performed in most cases.

No significant correlation could, however, be demonstrated between the hyaluronic acid changes and the systemic reactions in rheumatoid arthritis, contrary to the findings in group 5. This was further illustrated by subdivision of the groups of fig. 32 according to sedimentation rate, which showed a significant difference between the averages of intrinsic viscosity in the sub-groups of group 5 (10.5 ± 3.3; $P < 0.01$), whereas the corresponding difference in the rheumatoid group was only 3.7 ± 2.2. In the rheumatoid group there was, further, a close agreement between the sub-groups with regard to the averages of total protein and hyaluronic acid concentrations, as well as of relative viscosity and fluid volume, all results contrasting with the findings for sub-groups 5a and 5b.

The conditions in group 3 (degenerative joint disease) deserve brief mention. Nine cases exhibited moderately increased sedimentation rates (18—53 mm. per hour, average 30 mm.) which could not be otherwise explained by the clinical data available in most cases. The analysis data for this sub-group did not, however, differ from those of the whole group, the average intrinsic viscosity for the 9 cases being 29.1 against 29.8 for all the 42 cases of the group.

The relation of the intrinsic viscosity to fever is illustrated in table 35, for groups 4 and 5a.

Table 35. Relation of intrinsic viscosity to fever.

Groups	Fever present		Fever absent	
	No.	Intrinsic viscosity $M \pm \varepsilon (M)$	No.	Intrinsic viscosity $M \pm \varepsilon (M)$
4. Rheumatoid arthritis	7	23.1 ± 2.1	33	27.0 ± 1.4
5 a. Miscellaneous forms of arthritis with systemic reactions	8	19.0 ± 2.0	12	27.1 ± 2.1
Total	15	21.0 ± 1.5	45	27.0 ± 1.2

Difference between main sub-groups 6.0 ± 1.9 ($P < 0.01$)

The table shows that the average intrinsic viscosity was lower in the febrile cases, especially in group 5a. The significance of this finding is however difficult to assess in view of the small number of cases and the fact that presence of fever was as a rule also associated with a high sedimentation rate.

In conclusion it may be stated that some degree of correlation appeared to exist between the degree of hyaluronic acid changes in the fluids and the severity of systemic reactions.

In cases of rheumatoid arthritis Ragan and Meyer (1949) found the activity of disease to be roughly inversely proportional to a factor used as a measure of the mean polymerization of the fluid polysaccharide. Such a correlation could not, however, be demonstrated with certainty for the intrinsic viscosity test and the activity of the disease as reflected by an increased sedimentation rate.

Attempts to grade the activity of the disease more accurately by taking into account such factors as progressive joint involvement, anaemia, and so on, did not provide additional information. The clinical data were however not sufficiently uniform to permit reliable assessment of the activity.

Some further observations of interest in this connexion are given in tables 36 and 37 below.

In table 36 the relation of the intrinsic viscosity to some serological titres is illustrated.

It appears that in a small group of cases (all but two of rheumatoid arthritis) where streptococcus agglutination titres were available, increased titres were associated with a high degree of hyaluronic acid changes. If there is such a correlation, which could not be definitely settled on the basis of the present material, this should probably not

Table 36. Relation of intrinsic viscosity to serological titres.

The cases were subdivided according to serological titres, applying the conventional limits shown in the table.

Test	Titre	No. of cases	Intrinsic viscosity	
			$M \pm \varepsilon (M)$	Difference between sub-groups $D \pm \varepsilon (D)$
Streptococcus agglutination	$\leq 1/20$	10	30.5 ± 2.3	7.7 ± 3.3
	$\geq 1/40$	10	22.8 ± 2.4	$P < 0.05$
Antistreptolysin	< 200	25	26.4 ± 1.4	4.3 ± 3.2
	> 200	9	22.1 ± 3.4	

be taken as evidence of a streptococcal genesis of the hyaluronic acid changes. It would rather seem that both tests reflect the severity of the rheumatoid disease (see Boots *et al.*, 1952).

The corresponding results for the antistreptolysin test are also given in the table for a small series of cases of different types of arthritis. (Only two cases of rheumatoid arthritis showed titres > 200.) There was no close relationship between the results of the two tests.

In table 37 the relation of the variations of some of the chemical findings to changes in the erythrocyte sedimentation rate is shown for a case of severe rheumatoid arthritis under treatment with ACTH/cortisone. The fluids were obtained by aspiration of either knee at intervals of about 3 days during two courses of ACTH and one of cortisone therapy.[1]

As seen from the table, a favourable response to the treatment as indicated by the sedimentation rate tended to be associated with a decrease in protein content and increase in relative viscosity of the fluids. Similar findings have been reported previously by Clark *et al.* (1950), Kulonen (1951), and others. Clark *et al.* ascribed the increasing viscosity on ACTH administration to increased mean polymerization of the synovial polysaccharide, as judged from changes in the type of mucin precipitate (see chapter I, page 117). As the correlation coefficients in table 37 show, this could not be confirmed in the case studied. The results of a smaller series of analyses from a second patient were essentially the same, with no evidence of any correlation

[1] The response of the patient to ACTH was characterized as 'dramatic' on both occasions, whereas that to cortisone was less satisfactory (only 100 mg. daily was given).

I am indebted to Dr. Rolf Luft, Endocrinological Division of the Department of Internal Medicine, Serafimerlasarettet, Stockholm, for placing this material at my disposal.

Table 37. Correlation between erythrocyte sedimentation rate and synovial fluid findings during administration of ACTH/cortisone to a case of rheumatoid arthritis.

The rank correlation coefficients are given. *P* is the probability that the correlation is due to chance.

Correlation between erythrocyte sedimentation rate and–	No.	Rank correlation coefficient $\varrho \pm \varepsilon$ (ϱ)	*P*
Protein concentration	15	$+0.70 \pm 0.27$	<0.01
Hyaluronic acid concentration	15	-0.70 ± 0.27	<0.01
Relative viscosity	15	-0.55 ± 0.27	<0.05
Intrinsic viscosity	15	$+0.26 \pm 0.27$	

between the sedimentation rate and the intrinsic viscosity. Only insignificant correlation coefficients for protein and hyaluronic acid concentrations were, however, obtained in this case, the coefficient for relative viscosity being probably significant.

In conclusion it may be stated that the increase in viscosity of the synovial fluid occurring in immediate relation to ACTH administration is probably referable to increased hyaluronic acid concentration, which in turn is related to a decrease in degree of effusion rather than to changes in degree of polymerization of the polysaccharide.

X-RAY FINDINGS

The relationship between the degree of hyaluronic acid changes and the severity of disease as reflected by pathological radiograms was studied in the groups of degenerative and rheumatoid arthritis. Unfortunately a considerable number of cases had not been examined radiologically. Since the cases were collected from several different clinics, the principles employed in grading the radiological changes might have varied to some extent, and thus only a rough sub-divison of the cases according to radiological changes was possible.

Degenerative joint disease. The criteria used in grouping the cases of the degenerative group are apparent from table 38.

The fact that there was no difference in average intrinsic viscosity between the sub-group of 'early' and that of 'advanced' degenerative joint changes is some further support to the view (see pages 206 and 208) that the hyaluronic acid changes do not parallel the degree of osteo-arthritis, but are related to other factors.

Rheumatoid arthritis. The following radiological criteria were used in grouping the cases (see Steinbrocker *et al.*, 1949).

Table 38. Relation of intrinsic viscosity to X-ray findings in degenerative joint disease.

X-ray findings	Number of cases	Intrinsic viscosity $M \pm \varepsilon (M)$
Early degenerative changes[1]	18	28.8±2.0
Moderate or advanced degenerative changes	16	30.8±2.3
No data	8	30.1±3.3
Total	42	29.8±1.4

[1] The average of $[\eta]$ for 5 cases of 'chondromalacia patellae' included in this group was 24.9.

Group 1. No abnormality, osteoporosis only, or slight to moderate diminution of joint space, no bony erosions.
Group 2. Marked reduction of joint space in addition to bony erosions, present in all the cases, usually adjacent to the small joints of the hands.
Group 3. No data available.

A similar sub-division of the cases with regard to the degree of changes as revealed by radiograms of the knee joints only could not be made as the number of cases presenting 'advanced' changes according to the above definition was too small. The fluid findings in some cases showing radiological evidence of a varying degree of secondary degenerative changes in the knee joints were essentially the same as in the rest of the group.

As seen from table 39, the difference between the averages of intrinsic

Table 39. Relation of intrinsic viscosity to X-ray findings in rheumatoid arthritis.
For explanation of groups, see text.

X-ray findings	Number of cases	Intrinsic viscosity $M \pm \varepsilon (M)$
1. Slight to moderate changes	18	30.1±1.9
2. Advanced changes	16	22.9±1.8
3. No data	6	24.0±0.9
Total	40	26.3±1.2
Difference of M between sub-groups 1 and 2		7.2±2.6 ($P<0.01$)

viscosity of the sub-groups 1 and 2 is significant. In conclusion it may be stated that, *regardless of the site of the lesion*, the cases of rheumatoid arthritis showing advanced radiological changes also show the highest degree of alterations in the state of the hyaluronic acid of the knee joint effusions.

The conditions in the traumatic groups further warrant brief mention, especially with regard to the fact that several cases of uncomplicated traumatic arthritis following acute sprains had not been examined radiologically. However, the average intrinsic viscosity for these 14 cases was 39.4 against 36.9 for the remaining 26 cases of group 1, the difference being insignificant. Among the cases collected in group 1b were 6 cases of suspected lesions of the semilunar cartilages, where arthrography had either not been performed or the result had been considered as possibly indicative of slight damage to the semilunar cartilages. The average intrinsic viscosity for the 6 fluids was 33.8 against 37.5 for the remaining 14 cases of group 1b. Finally, among the 15 cases of group 2 of traumatic arthritis associated with confirmed lesions of the menisci, there were 4 instances where the radiograms revealed a varying degree of degenerative change. The average of $[\eta]$ for these cases was 36.1 against 30.1 $\pm$ 1.8 for the whole group.

In summarizing the results for the traumatic arthritis groups it may thus be stated that a more careful selection of the cases with regard to X-ray findings would apparently not have influenced the results to any extent.

The radiological findings in group 5 of arthritis of infectious or unknown aetiology were too variable to allow of more detailed analysis of the cases in this respect.

EXTENT OF JOINT INVOLVEMENT

Part of the material was studied with respect to possible relationships between the degree of hyaluronic acid changes in the joint fluids and the tendency to multiple joint involvement. Reliable clinical data were available in most cases in the rheumatoid group. Since the clinical examination had not been specially adapted for detecting slight changes or signs of disease in other joints than that which was the site of the actual disability, the results given below for groups 3 and 5 are presented with circumspection.

Degenerative joint disease. In 17 of the 42 cases the clinical records indicated degenerative changes in other joints. The average intrinsic viscosity in this sub-group was 32.0 $\pm$ 2.3 against 28.4 $\pm$ 1.7 for the rest of the group, the difference of 3.6 $\pm$ 2.9 being insignificant.

Rheumatoid arthritis. The average of $[\eta]$ in a small group of 5 cases with involvement of only a few large joints was 33.1 against 24.8 for 31 cases where peripheral small joints were also involved. In the remaining 4 cases of group 4 no reliable data regarding the extent of joint involvement were available.

Arthritis of infectious or unknown aetiology. Of the 20 cases with systemic reactions (sub-group 5a) there were 8 of unclassified arthritis where signs of disease in more than one joint were present at the time

of aspiration, or developed soon after.[1] The average intrinsic viscosity for this sub-group was 27.8 as compared with an average of 23.9 ± 1.8 for the whole group. In the 'unclassified' group, 5b, the disease appeared to be localized to the joint aspirated except for two cases of bilateral involvement of the knee joint.

Although the numbers were too small to allow of definite conclusions, it may be stated that the hyaluronic acid changes apparently bore no relationship to the severity of disease as reflected by the extent of joint involvement, with the possible exception of rheumatoid arthritis. The significance of the finding of only slight hyaluronic acid changes in the fluids of a small group of 'atypical' cases of rheumatoid arthritis not presenting involvement of peripheral small joints could not however be further analysed on the basis of the present material.

CELL COUNTS

Although cell counts were only available in a very limited number of cases it seemed of interest to investigate whether the trend of the material indicated any relationship between the chemical tests and the number of leucocytes present in the fluids. Of the common tests, the sugar difference between fluid and plasma, and the total protein concentration of the fluid, are known to parallel roughly the increase in number of white cells (see Kling, 1938).

Sub-division according to the total number of white cells per mm^3 fluid of a group of 22 cases of miscellaneous arthritic conditions showed that in the sub-group with high counts ($> 9\,000$ cells per $mm.^3$) the averages of hyaluronic acid and relative viscosity were lower, and those of total protein concentration and fluid volume higher, than in the sub-group with low counts ($< 5\,000$ cells per $mm.^3$), whereas the averages of intrinsic viscosity were essentially the same.

By the rank method the correlation coefficients for protein content and relative viscosity were found to be probably significant, whereas insignificant or no correlations could be demonstrated for hyaluronic acid concentration, intrinsic viscosity, or fluid volume.

SUMMARY OF CHAPTER IX

The results obtained in the analysis of the relation of the different tests to various clinical and laboratory data are summarized below for the different groups of arthritic disease.

[1] Probably some of these patients actually suffered from early rheumatoid arthritis (see Egelius *et al.*, 1949).

Traumatic arthritis. The great variation of the values of protein and hyaluronic acid concentrations in the fluids of this group can be referred to the varying degree of effusion, the protein concentration being directly, and the hyaluronic acid concentration and relative viscosity inversely, proportional to the fluid volume. Qualitative changes in the hyaluronic acid were essentially absent except in those cases where lesions of the semilunar cartilages were present, and in some cases where such lesions had been suspected although not definitely proved. In both instances the degree of changes, as revealed by moderately to greatly decreased intrinsic viscosity, was most marked in effusions aspirated shortly after the injury which had presumably caused the meniscus lesion. Although some degree of degenerative joint change can be expected in older patients, it was not possible to demonstrate any relation of the intrinsic viscosity test to the age of the patients among the cases in this group.

Degenerative joint disease. No significant relation of the fluid findings to such factors as sex and age, degree of effusion, duration of joint disability, or severity of disease, could be demonstrated. The great variation of intrinsic viscosity found in the fluids of this group can obviously not be ascribed to the heterogeneity present with respect to the above factors. It is concluded that the hyaluronic acid changes in the joint fluids are unrelated to the degree of degenerative joint change *per se,* and must be ascribed to other factors.

Rheumatoid arthritis. No obvious relation of the fluid findings to sex- and age-factors, degree of effusion, duration of disease, or of knee joint disability, could be demonstrated. Cases presenting marked constitutional reactions, such as fever and highly increased sedimentation rate, showed on an average lower values of intrinsic viscosity. There was, however, no significant correlation between the intrinsic viscosity and the activity of disease as reflected by the sedimentation rate.

The increasing fluid viscosity, roughly in inverse proportion to the fall in sedimentation rate, observed in 2 patients with severe rheumatoid arthritis under treatment with ACTH/cortisone could hardly be ascribed to restoration of the intrinsic viscosity to normal, but it closely paralleled changes in the polysaccharide content of the fluid. The protein concentration varied on the other hand approximately in direct proportion to the sedimentation rate.

Increased streptococcal agglutination titres appeared to be associated with marked hyaluronic acid changes in a small series analysed.

The degree of changes of the fluid polysaccharide apparently did not solely depend on the degree of local changes in the aspirated knee joint, but paralleled the extent and severity of joint involvement, in that the lowest intrinsic viscosities were found in those cases where the radiograms revealed the disease to be extensive and in an advanced stage, regardless of the site of the lesions.

In a small group of 'atypical' cases where the disease was restricted to a few large joints, only moderately lowered values of intrinsic viscosity were found.

Arthritis of infectious or unknown aetiology. Regardless of the aetiology of the arthritis, cases presenting systemic reactions (subgroup 5a) also showed a high degree of change in the fluids, which was most marked in acute febrile arthritis.

The difference with regard to most fluid findings shown to be present between sub-groups 5a and 5b, could not be ascribed to different distributions of the cases between the sub-groups with respect to such factors as sex, age, or duration of disease.

The decrease in intrinsic viscosity of the fluid hyaluronic acid was roughly proportional to the activity of disease as reflected by the sedimentation rate. In this group, contrary to the findings in the rheumatoid and degenerative arthritis groups, the increase in protein concentration was found to parallel the degree of effusion.

Those cases where there was a tendency to multiple joint involvement did not differ from the rest of the group with regard to the fluid findings.

The hyaluronic acid changes did not appear to parallel the number of white cells present in the fluids in a small series of different types of effusions.

CHAPTER X

Some Remarks on the Pathogenesis of the Hyaluronic Acid Changes and their Bearing on the Physiological Function of the Joint Fluid

A lowered degree of polymerization of the synovial hyaluronic acid in disease may be due either to the presence of some factor which depolymerizes the normal substance, or to a disturbance of its normal synthesis, or to both. There is no positive evidence in support of the first; neither active hyaluronidase nor low-molecular degradation products have been found in pathological joint fluids. It has been suggested that alterations in factors known to depolymerize hyaluronic acid *in vitro*, such as ascorbic acid or alkaline phosphatase, influence the polysaccharide in disease (Ropes *et al.*, 1947; Teilum, 1952; and others), but so far without actual experimental support. In the lack of positive evidence of the presence of a depolymerizing agent, disturbed synthesis has appeared as the more plausible explanation. The end-result of the disturbed polysaccharide metabolism in rheumatoid arthritis has been characterized by Ragan and Meyer (1949, 1950) as an overproduction of incompletely polymerized hyaluronic acid.

The present investigation has shown that a subnormal degree of polymerization of the hyaluronic acid is not specific of rheumatoid arthritis, but occurs in most joint diseases, the only outstanding exception being fresh effusion after acute trauma. A question of prime importance is, then, whether a common denominator can be found to explain hyaluronic acid changes in diseases of greatly varying aetiology and nature.

The marked decrease in intrinsic viscosity found in fluids aspirated in close relation to meniscus lesions (table 33, page 208) and in most cases of degenerative joint disease (fig. 26, page 191) suggests that acute damage to the cartilage may be of pathogenetic importance. Destruction of cartilage is also a common feature of various types of arthritic disease.

It seemed unlikely that the observed decrease in intrinsic viscosity of the hyaluronic acid might, in some cases, be only apparent and due to liberation of low-viscous polysaccharides from the cartilage, and this possibility was further ruled out by the failure to demonstrate the presence of such polysaccharides in these fluids (see discussion in chapter VI).

No close relationship was indicated between the degree of hyaluronic

acid changes in the fluids and the stage or severity of degenerative joint changes (see summary, page 221).

In view of this, a likely explanation of the hyaluronic acid changes in these fluids would be that they reflect reactions of the synovial tissue in response to trauma and irritation by the damaged cartilage.

This hypothesis is supported by the finding by Soeur (1949) of marked 'synovial activity', predominantly proliferation of villi and fibrosis, at the onset in cases of meniscal tears. The changes in the synovial membrane were found to subside after some months, and thus they roughly parallel the occurrence of hyaluronic acid changes in the fluids reported here.

Similar changes are known to occur also in degenerative joint disease. In analogy with the observed hyaluronic acid changes in the fluids (see page 221), the extent of changes in the synovial membrane has been reported to parallel neither the degree of alterations in the other articular structures (Bennett *et al.*, 1942) nor the duration of symptoms (Ghormley *et al.*, 1952) in this disease.

Bick (1930) states that hypertrophy and proliferation of villi are to be regarded as an entirely non-specific tissue reaction to more or less persistent irritation, whether of infectious, allergic, or traumatic origin. It seems likely that the degree of hyaluronic acid changes in the fluids simply reflects the extent of connective tissue proliferation in the synovial and peri-articular tissues. It may well be that newly formed granulation tissue produces a hyaluronic acid of subnormal degree of polymerization.

In the discussion of the viscosity changes occurring in some fluids on incubation at 37° C. (see page 203) it was stated that the final polymerization processes probably take place extracellularly in the peri-articular connective tissue. A possible explanation of the lowered intrinsic viscosity in pathological fluids would then be accelerated release of incompletely polymerized compounds associated with exudation into the joint cavity. There was, however, no obvious relationship between intrinsic viscosity and fluid volume or protein content of the fluid, as might have been expected if the above hypothesis were correct. Marked decrease in intrinsic viscosity was further present in several cases in spite of insignificant exudation.

Unless compensated by increased rate of turnover of the hyaluronic acid, the connective tissue proliferation should result in an increase in hyaluronic acid content of the joint cavity. Taking the incidence of incompletely polymerized polysaccharide as an indication of proliferative changes, an inverse relationship between the values for intrinsic viscosity and for 'total hyaluronic acid' (see page 188) may be expected.

Such a tendency is apparent from fig. 32, but correlations of significant degree were not present in any of the groups of arthritis 1–5.

Plotting the intrinsic viscosity against the unit concentration of hya-

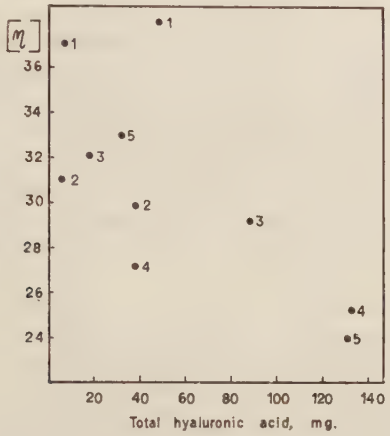

Fig. 33. Diagram showing the relationship between changes in intrinsic viscosity and total hyaluronic acid content of pathological synovial fluids.

The numerals indicate groups of arthritis as defined in page 181. The groups were subdivided according to total hyaluronic acid, the averages in the sub-groups being given. Rank correlation coefficient for the 10 couples $= -0.66 \pm 0.33$ ($P < 0.05$).

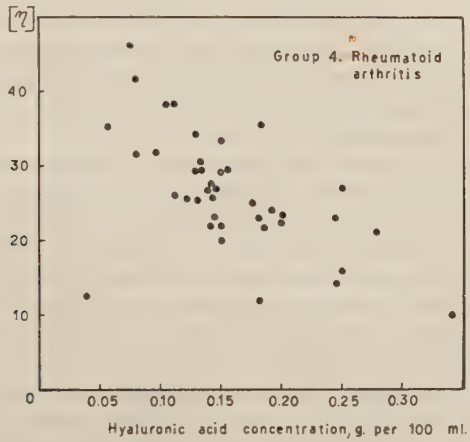

Fig. 34. Changes in intrinsic viscosity and hyaluronic acid concentration in fluids from rheumatoid arthritis (group 4).

See also table 40.

luronic acid (see fig. 33), an inverse relationship seemed to be present in some groups.

In this respect the rheumatoid group differed significantly from the traumatic arthritis cases (see table 40). The same tendency was present also in groups 3 and 5.

Table 40. *Relationship between changes in hyaluronic acid concentration and in intrinsic viscosity in fluids from different types of arthritis.*

The correlation coefficients between intrinsic viscosity and hyaluronic acid concentration were compared in groups 1–5 as defined in page 181 (see also fig. 34). Fisher's z-transformation (see Fisher, 1948, page 229) was used in calculating the values of P given.

Group	No. of cases	Coefficient of correlation	P for deviation from coefficient in group 1
1. Traumatic arthritis	40	−0.08	
2. Lesions of the menisci	15	+0.02	
3. Degenerative joint disease	42	−0.41	
4 Rheumatoid arthritis	40	−0.63	<0.01
5. Arthritis of infectious or unknown aetiology	40	−0.51	<0.05

The explanation of these findings is not obvious. It is possible that increased hyaluronic acid production occurs as a kind of regulative reaction for the maintainance of the viscosity, and thus the essential lubricating properties of the fluid.

Marked deviation from the conditions in groups 3–5 was found in fluids aspirated at varying times after surgical interference (see table 26, page 197). Marked decrease in intrinsic viscosity was thus associated with low values for hyaluronic acid concentration. No explanation of the hyaluronic acid changes in these fluids can be offered.

No essential difference in type of pathological fluid findings could be demonstrated between rheumatoid arthritis and other arthritic conditions where tissue inflammation is presumably of about the same degree. The further analysis of the cases, presented in chapter IX, revealed, however, some discrepancies between the rheumatoid arthritis group and group 5a, consisting of miscellaneous forms of usually acute arthritis with systemic reactions. Thus, whereas in the latter group the hyaluronic acid changes roughly paralleled other signs of tissue inflammation in the aspirated joint, such as degree of effusion and increase in total protein in the fluids, no such parallelism could be demonstrated in the cases of rheumatoid arthritis. This difference, however, may well be explained by disturbed removal of colloids and fluid from the joint cavity in chronic arthritis (see Bauer *et al.*, 1940; Edlund, 1949; Gardner, 1950).

Further, in rheumatoid arthritis there was no close relationship between the degree of fluid changes and of systemic reactions, in contrast with the conditions in group 5a.

The finding (see table 39, page 218) of significantly lower average intrinsic viscosity in a sub-group of cases where radiograms showed the rheumatoid arthritis to be in an advanced stage, seemed notable. On subdivision of the group, the *sites* of the lesions were thus not considered. No gross difference with respect to degree of radiological changes in the aspirated joint was on the other hand present between the subgroups.

Although inconclusive, the data recorded above seem to suggest the possibility that in rheumatoid arthritis the decrease in intrinsic viscosity in the fluids may not solely be an indication of local changes in the connective tissue surrounding the aspirated joint, but also of a general change in the hyaluronic acid of the body.

The idea of a generalized type of hyaluronic acid change in some cases of degenerative joint disease warrants consideration, since such a disturbance might well be thought to account in part for an early appearance of the disease by reducing the lubricating power of the joint fluid. The inheritance of qualitatively inferior cartilage as a factor of importance in this connexion has been suggested by several workers (Bauer and Bennett, 1936; and others) and might be ascribed to a disturbance of the metabolism of the chondroitin sulphate of the cartilage, a polysaccharide which is closely related to hyaluronic acid. A falling polysaccharide content of the cartilage with increasing age, presumably accounting for loss of resilience and elastic strength, has in fact been reported to precede the appearance of degenerative joint changes (see reviews by Hirsch, 1944; Sylvén, 1947). These changes can, however, in most cases probably be ascribed to the fact that the cartilage is avascular and accordingly sensitive to nutritional disturbances. So far there seems to be no experimental evidence supporting the view that a primary disturbance in the polysaccharide metabolism may occur as a contributory factor.

As pointed out above, the marked variation in intrinsic viscosity in the degenerative arthritis fluids could not be accounted for otherwise than by assuming that it was due to a varying degree of secondary reactions in the synovial membrane. It was not possible, however, to demonstrate any close relationship between the degree of hyaluronic acid changes and such evidence of tissue inflammation as degree of effusion or protein content of the fluids.

The possibility may then be considered, that part of the variation in fact arises from a variability unassociated with local changes in the aspirated joint. In support of this was the marked decrease in intrinsic viscosity, found in 5 out of 9 cases from groups 2–3, where the amount of fluid present in the joint cavity was only insignificantly increased (below

4 ml.). Some evidence of tissue inflammation was, however, present also in these cases, since the average protein concentration was 4.4 g. per 100 ml. as compared with 2.5 g. per 100 ml. in a group of normal or probably normal fluids of similar fluid volume.

Actual experimental evidence supporting a primary disturbance in the hyaluronic acid metabolism in degenerative joint disease could thus not be provided. However, the influence of hyaluronic acid changes, of the degree revealed by the intrinsic viscosity test in this investigation, upon the lubricating ability of synovial fluid warrants brief consideration. The discussion below may in part be relevant also for other types of joint disturbance.

Previous experimental investigations (see Ropes *et al.*, 1947; Gardner, 1950, for references) have not provided conclusive evidence of insufficient lubricating properties of pathological synovia. Ropes *et al.* found the ability of the fluids to reduce static friction to be essentially the same in all types of joint fluids in experiments using a model joint. They also failed to demonstrate that deficient stability of the lubricating films might be a factor of importance, to judge from tests performed with pathological fluids of widely varying viscosity. The bearing of the latter results upon the conditions which may actually be encountered *in vivo* could, however, not be definitely established.

Gardner (1950) in a review on the physiology of movable joints, has drawn attention to the fact that the lubrication encountered *in vivo* is usually not of the same type as that in rigid models. Tests in artificial joints may give information only about the lubrication between what are in principle parallel surfaces.

In vivo, however, the articulating surfaces are congruent with each other only to a limited extent during motion. Solid or boundary lubrication is therefore not the rule, but may occur at rest, in the outer parts of the motion range, and on sudden rapid movements.

Usually there are only partial, and continuously varying, potential contacts between the articulating surfaces during motion. The incongruency between the ends is in fact of great importance in lessening the amount of solid lubrication, which implies a greater risk of damage to the cartilage. The lubrication is on the other hand mainly achieved by wedgelike fluid films which undergo continuous displacement from one part of the cavity to another, thereby filling out the changing space between the surfaces.

All factors which interfere with the formation of fluid films will thus reduce the efficiency of lubrication.

A decrease in synovial fluid viscosity will impair the ability of the fluid film to resist shear, and may extend the solid lubrication into phases of movement. The stability of the boundary layer separating the surfaces in the latter type of lubrication is probably also affected by a reduction in viscosity.

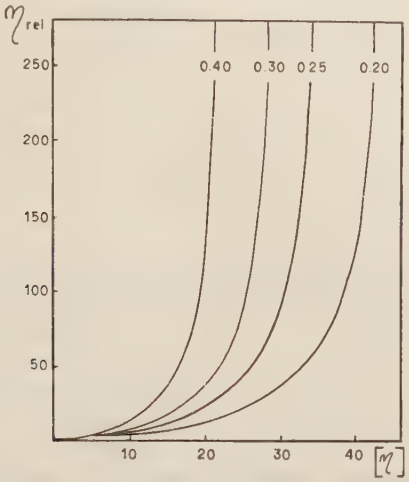

Fig. 35. Diagram showing the relation of the relative viscosity of synovial fluid to the intrinsic viscosity at constant hyaluronic acid concentration.
Numerals indicate hyaluronic acid concentrations in g. per 100 ml.

Even slight changes in intrinsic viscosity of the hyaluronic acid will greatly influence the η_{rel} of the fluid in the range of concentrations probably encountered in non-arthritic joints (see fig. 35).

This is further illustrated by the finding of an average relative viscosity, as calculated from the analysis data, of about 40 at 37° C. in a group of 9 fluids from degenerative joint disease and traumatic arthritis associated with lesions of the menisci, whereas the corresponding value in a group of 10 normal or probably normal fluids was 235. The fluid volumes were approximately within the normal range in all cases (below 4 ml.), with averages of 2.0 and 1.8 ml. respectively. There was no evidence of any 'compensatory increase' in polysaccharide concentration in these pathological fluids (see page 226).

The pronounced anomalous behaviour of the viscosity of normal fluid may also be essential for its lubricating properties. The viscosity will thus be high at low rates of motion but will decrease rapidly at faster rates. It seems that this may be of importance in promoting the stability and smoothness of motion. The ability of the fluid film to resist a load can further be expected to be considerably increased by the high tendency to particle aggregation even at low concentrations of polysaccharide.

Since the degree of flow anomalies increases rapidly with increasing degree of polymerization (see chapter IV, figs. 8–10), it follows that a lowered intrinsic viscosity of the hyaluronic acid may well impair the lubricating properties of the fluid, even if the viscosity can be maintained at a constant level by an increase in concentration.

General Summary

The main object of this investigation has been to study quantitatively and qualitatively the hyaluronic acid in normal and pathological synovial fluids (chapter I).

The general methods employed are described in chapter II. The material investigated included protein-free preparations of hyaluronic and chondroitin sulphuric acid, 24 joint fluids from animals, and 250 fluids from the knee joints of 206 patients.

In chapter III a simple method is described, which allows direct determination of the glucosamine component of the hyaluronidase-sensitive polysaccharide in 0.1–0.2 ml. of synovial fluid.

The state of the naturally-occurring polysaccharide was studied by comparison of the viscous behaviour of synovial fluids with that of solutions of isolated hyaluronate (chapter IV). Equations are given based on studies of the relationship between viscosity and concentration over a wide range of viscosities, and valid both for hyaluronate solutions and naturally-occurring fluids. Using these equations the intrinsic viscosity of the polysaccharide may directly be calculated from a single determination of relative viscosity, provided the concentration is known.

The influence upon the values of intrinsic viscosity of variations in the measuring conditions was further studied, with regard to temperature, ionic strength of solvent, and rate of flow in the capillary.

Marked differences in degree of anomalous viscosity were observed between solutions of pure hyaluronate and naturally-occurring fluids. These are ascribed to the variation in degree of polymerization of the polysaccharide. A method is described by which comparison of intrinsic viscosities can be made using viscometers of fixed dimensions.

The nature of the synovial polysaccharide has been further studied by means of analysis of degradation curves obtained after addition of a constant amount of testicular hyaluronidase to substrates of joint fluids or pure hyaluronate solutions (chapter V). The basis for this analysis was a new method, by which the degradation rate can be calculated directly from two viscosity determinations over an arbitrarily chosen time interval in the initial stage of the enzymatic hydrolysis.

In experiments using pure hyaluronates this degradation rate was found to be independent of the initial viscosity of the substrate within wide limits.

The procedure described provides a simple method of determining hyaluronidase activity, its greatest advantage being that reproducible results are obtained with hyaluronates of varying mean polymerization.

The apparent degradation rate was greatly decreased in the presence of even small amounts of chondroitin sulphate.

In the experiments with synovial fluids as substrate the shape of the degradation curves was found to be similar to that obtained for solutions of isolated hyaluronate. The results obtained on comparison of relative degradation rates in a series of synovial fluids of all types tended to exclude the presence in the fluids of significant amounts of hyaluronidase-sensitive, sulphated polysaccharides. In keeping with this were the results of a few analyses of ester-bound sulphate performed on joint fluids of different types.

The results of the viscosity studies, discussed in chapter VI, gave strong support to the view that the much higher viscosity at unit concentration observed in normal synovial fluid, as compared with solutions of isolated hyaluronate, can be ascribed entirely to higher mean polymerization of the naturally-occurring polysaccharide.

On the basis of the results reported for pure hyaluronate preparations by Blix and Snellman (1945), molecular weights of the order of several million were indicated for the hyaluronic acid of normal synovial fluid.

Determinations of intrinsic viscosity seemed further to provide a reliable method for indicating changes in degree of polymerization of the hyaluronic acid occurring in pathological joint fluids.

The routine analysis of the synovial fluids included, in addition to determinations of hyaluronic acid concentration and intrinsic viscosity, a test of the stability of the viscosity at 37° C., and determination of total protein concentration. The last-mentioned tests were, however, only performed on part of the series.

The analysis results for the normal fluids of human and animal origin are given in chapter VII. The hyaluronic acid concentration and the relative viscosity were found to vary within wide limits, approximately in inverse proportion to the fluid volume. The values of intrinsic viscosity and of protein concentration were less variable and bore no obvious relation to the fluid volume.

Although the composition of the fluid in any particular joint probably varies according to the functional state, fluid from the normal human knee joint may be expected to contain about 0.2–0.4 g. hyaluronic acid and about 2–3 g. protein per 100 ml. With levels of intrinsic viscosity above about 40, as found for the highly polymerized polysaccharide of normal fluid, this range of hyaluronic acid concentrations would correspond to a minimum relative viscosity of several hundred.

The main part of the series of pathological fluids was arranged into 4 groups (chapter VIII), as follows. 1. Traumatic arthritis. 2. Traumatic arthritis associated with lesions of the menisci. 3. Degenerative joint

disease. 4. Rheumatoid arthritis. 5. Arthritis of infectious or unknown aetiology.

The more important chemical differences between fluids from the different groups of arthritis are summarized in table 24 (page 192), where the possible diagnostic value of the tests is also discussed.

The hyaluronic acid concentration and relative viscosity were found to vary greatly in all types of fluids, and thus, with few exceptions, these tests were found to be of very limited value in differentiating between the groups. The concentration of total protein was found to be more informative, as was also the approximate total hyaluronic acid content of the fluid obtained from the hyaluronic acid concentration and total volume. Both types of change apparently increase with increasing severity of tissue inflammation, the highest levels being found in severe rheumatoid or infectious arthritis.

Although the above tests may all be of some aid in differentiating fluids of rheumatoid or infectious arthritis from those of traumatic disorders or degenerative joint disease, they are of no definite value in distinguishing between the two last-mentioned types of joint disturbance. Marked differences were on the other hand found by means of the intrinsic viscosity test. In the fluids of traumatic arthritis qualitative hyaluronic acid changes were thus essentially absent, with the exception of fluids aspirated within some weeks of injuries associated with intra-articular cartilage lesions. Moderate to marked decrease in intrinsic viscosity was on the other hand observed in most fluids of degenerative joint disease. Marked hyaluronic acid changes were further found in the majority of cases of rheumatoid or infectious arthritis, the lowest values of intrinsic viscosity being found in cases of acute, febrile arthritis, regardless of the aetiology.

The intrinsic viscosity test thus seems to be of definite value in detecting underlying joint disease in cases of supposed traumatic arthritis (see diagram, page 191).

The converse is, however, not true, since normal values may be found in all types of effusions, although rarely in rheumatoid and infectious arthritis. Although the test is of no value *per se* in differentiating infectious or rheumatoid arthritis from degenerative joint disease, the combination of findings may be of diagnostic value.

The fluid findings in miscellaneous types of joint disturbance are further briefly discussed in chapter VIII. In the last part of that chapter the results of studies on the stability of the viscosity are given. Both in the fluids of traumatic arthritis and of degenerative joint disease a significant increase in viscosity was observed on incubating the fluids at 37° C. In the fluids of rheumatoid or infectious arthritis, on the other hand, only insignificant changes occurred under the same conditions. The increase in viscosity can be considered as probably due to enzymatic polymerization of the polysaccharide (see discussion, page 202).

An attempt to analyse the possible relationship of the fluid changes to various factors is presented in chapter IX. Regarding the smallness of the series, some of the results summarized in page 220 should not be regarded as conclusive.

With few exceptions the hyaluronic acid changes bore no obvious relationship to sex and age, degree of effusion, or duration of disease.

There was no evidence of any parallelism between the changes in intrinsic viscosity and the degree of radiological changes in degenerative joint disease.

In rheumatoid arthritis the hyaluronic acid changes were most marked in cases where radiograms revealed the disease to be extensive and in an advanced stage, regardless of the site of the lesion. This may indicate that the polysaccharide changes in the fluids do not solely depend on the severity of the *local* changes in the joint in this disease. There was, however, no close relationship between the degree of fluid changes and the activity of disease as reflected by the sedimentation rate.

The nature of the hyaluronic acid changes observed in pathological conditions is discussed in chapter X. The possibility is considered that the changes in the synovial polysaccharide occurring in rheumatoid arthritis and degenerative joint disease are an indication of a general change in the hyaluronic acid of connective tissue. Finally, the bearing of the hyaluronic acid changes upon the lubricating properties of the synovial fluid is discussed.

Acknowledgements

I would like to thank Professor Gunnar Blix for his constant help and advice, and for placing the polysaccharide preparations at my disposal.

My thanks are further due to the Chiefs and to members of the staffs of a number of clinics for supplying the clinical material, and for advice and criticism.

I am greatly indebted to Mr. Evert Lindberg for help with the analytical procedures, Miss Ulla Lindberg and Mr. Knut Lundin for technical assistance, Mrs. Barbro Hedlund for secretarial aid, Mr. Nils Blomqvist, Fil. lic., for advice concerning statistical problems, and Dr. Marcia Skogh for the revision of the English text.

The work has been supported by grants from the Swedish State Fund for the Promotion of Medical Research and from the Regnell Foundation of the Medical Faculty at Uppsala.

Statistical Symbols

M = arithmetic mean
$\varepsilon(M)$ = standard error of the mean
D = difference
$\varepsilon(D)$ = standard error of the difference
σ = standard deviation
σ_i = error of a single determination calculated from series of duplicate determinations
V = coefficient of variation
n = number of observations
P = significance level
t = t according to 'Student' (see Fisher, 1948)
r = coefficient of correlation
$\varepsilon(r)$ = standard error of the coefficient of correlation
ϱ = rank correlation coefficient
$\varepsilon(\varrho)$ = standard error of the rank correlation coefficient

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Abbreviations after Quarterly Cumulative Index Medicus.

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